STUDIES ON THE ADSORPTION OF FILAMENTOUS BACTERIOPHAGE IKe.

by

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ABSTRACT

IKe, the filamentous bacteriophage with a N-type plasmid specificity, attaches to its host cell at a rate constant of 23×10^{-11} $cm^3 \, min^{-1}$. Diffusion appears to be the rate limiting factor only up to a certain maximal cell density, at which a saturation point is apparently reached and the rate of adsorption no longer increases linearly as cell concentration increases.

The presence of 0.1M NaCl in the medium is required for maximal levels of adsorption while the addition of $CaCl_2$ does not affect attachment. In media of ionic concentrations optimal for attachment of the phage to its host cell, binding of the phage to non-biological surfaces of net negative charge (membrane filter discs) can also be demonstrated.

Adsorption is optimal at temperatures ranging from 30 to 40°C and within the pH range of 5-8. In a medium containing an excess of H^+ cations (pH 4.2 or lower) specific adsorption is greatly reduced and replaced by a non-specific type of binding. Low levels of attachment are obtained at 0°C, in starvation media or when the metabolic activity of host cells is otherwise reduced (i.e. cells in the stationary growth phase or in the presence of metabolic poisons).

It seems likely therefore that the first step in the infection by phage IKe, although it is apparently mediated by the formation of electrostatic bonds, is considerably dependent on and determined by the physiological state of the host cell.

Additional findings suggest that the phage attaches irreversibly to a receptor on the N-plasmid bearing cell which (unlike the sex pilus associated receptor) cannot be removed by mechanical agitation. The irreversible attachment of IKe is followed by a step of penetration. The rate of penetration is rapid in the first 10 minutes of infection and decreases thereafter.

CHAPTER I

INTRODUCTION

History and structural properties of filamentous bacteriophages

Filamentous bacteriophages were first isolated by Loeb in 1960 (46). Their structure as well as their mode of infection have since been extensively studied and have comprised the subject of several reviews (4, 10, 12, 48, 69).

More than 30 filamentous phages had been described by 1969 (48) and since then more have been isolated (38). However, most of the information now available pertaining to this group of bacteriophages has been obtained in the course of study of M13, fd, f1, If1 and If2 (32, 46, 47, 52, 70, 83, 85). All filamentous phages presently known share several structural properties: Their DNA is single stranded and present in the particle in a circular form (48). Two components of their coat protein have been described these being the A and the B proteins. The former is a minor protein located at one end of the particle. It has been implicated in phage adsorption and is also believed to penetrate the bacterial cell and play a role in phage replication. The B protein comprises the bulk protein and its properties have been studied in several laboratories (35, 35, 44, 48, 49, 73).

The structure of the filamentous phage is not yet fully understood. Two models have been considered, on the basis of diffraction patterns and electron microscopy of the viruses. One model proposes a cylinder shaped particle with the DNA located in an axial hole down the middle of

the cylinder. The other suggests two parallel cylinders each with a central single stranded DNA (25, 29, 48). Both models are still under investigation (49).

All known filamentous coliphages have been shown to specifically adsorb to bacterial cells carrying a conjugative plasmid. This includes Hfr cells in which the F (fertility) plasmid exists in a chromosomally integrated form and not as an autonomous plasmid. (48).

The specificity of filamentous bacteriophages

Since filamentous bacteriophages owe their specificity to the plasmids harboured by their host cells, their classification could be expected to parallel the taxonomical grouping of plasmids. On this consideration the filamentous phages studied so far (with the exception of phage IKe, see p. 9) have been grouped as follows: (48, 53).

1. The F-specific (or Ff) bacteriophages: This group consists of the filamentous bacteriophages which attach specifically to cells bearing a F-like plasmid. These include F plasmids and those R (resistance) and col (colicin) plasmids which repress F-associated functions when introduced into F-or F'-plasmid bearing cells (F^+ or F'^+ cells). These plasmids were originally designated fi^+ (fertility inhibition positive) (81) a designation which has been recently replaced by Fi^+ (F) (56). The best studied bacteriophages in the Ff group are M13, fd, and f1.

2. The I-specific (or If) bacteriophages: Bacteriophages in this group attach specifically to cells carrying an I-like plasmid. Among the latter

are those colicin and R plasmids which do not repress F-associated fertility in a F^+ cell and have been designated fi^- or Fi^- (F).

If1 and If2 are the only known bacteriophages in the If group. It is possible that these two phages, though isolated on separate occasions, are identical since their sera were found to cross neutralize (41). They were therefore referred to collectively as phage If (38).

Attachment of the filamentous bacteriophages

Bacterial cells carrying either F-or I-like plasmids have been shown to synthesize a specific pilus distinct from other surface appendages generally associated with the enterobacteria. This organelle has been implicated in the specific conjugal transfer of the plasmid and was therefore designated the sex pilus (13, 51). Sex pili synthesized by cells carrying I-like plasmids have been shown to be both morphologically and antigenically different from the pili synthesized by cells carrying F-like plasmids (41). It should be noted however that although structurally and antigenically similar, several serotypes have been distinguished within the two classes of sex pili (42).

The receptor site for the filamentous bacteriophage is generally believed to be located on the tip of the sex pilus. The adsorption of the phages to the tip of pili either protruding from the cell surface, or detached and free in the media was demonstrated by electron microscopy (15, 52). Several lines of evidence (derived mostly from work with F or F-like plasmid bearing cells) support the inference that the sex pilus

which is involved in the conjugal transfer of plasmid DNA is also required for the adsorption of the plasmid-specific filamentous phages. This evidence is as follows:

(1) A change in the number of microscopically visible pili on a host cell culture causes a corresponding and co-ordinate change in the ability of such cells to conjugate as well as to adsorb the donor-specific bacteriophages. The changes in the number of pili on the cell surface can be caused by one of the following factors:

(a) the growth phase of the host strain (10).

(b) repressed or derepressed state of the transmissible plasmid (associated with wild type or mutant state of the plasmid regulatory genes which determine the synthesis of a cytoplasmic repressor) (50).

(c) growth of donor strain in the presence of 5-bromouracil (76).

(d) mechanical agitation of host cells which results in removal of the sex pilus (61).

(2) The attachment to donor cells of the specific filamentous bacteriophages blocks or reduces their ability to transfer the plasmid to recipient cells (33, 60, 71).

(3) Both adsorption of the donor-specific phage and the transfer of the plasmid can be blocked by the pilus-specific antiserum (11).

(4) Both the frequency of conjugal transfer and the adsorption of specific filamentous bacteriophages to plasmid bearing bacteria, are co-ordinately reduced in the presence of Zn^{++} (63, 64).

Similar lines of evidence have also indicated that in addition to its role in conjugation and filamentous phage adsorption, the F pilus also provides receptor sites for the isometric RNA bacteriophages. Those phages have been shown to specifically attach along the sides of the F pilus (18, 33, 39, 60, 61, 76).

The number of pili on the cell surface has been shown to range in most cases from 1-3. Some cells however have been shown to carry as many as 30 pili (10, 15). Generally, one filamentous bacteriophage has been observed to attach to a single pilus, but electron micrographs have also revealed 2 and in rare cases 3 bacteriophages attached to some pili (15).

Since breakages of the pilus at any point along its entire length have been shown to create new sites for phage attachment at the broken sites, it is assumed that attachment sites for the filamentous phages are actually located along the length of the pilus, but made available only when breakages occur and (broken) tips are exposed (15).

The role of the pilus in the infection by filamentous (as well as isometric RNA) bacteriophages is still a matter of controversy. According to one proposed model (the conduction model), the pilus serves as a conductor for phage DNA which enters the host cell following phage attachment (10, 11). Several recent reports however, seem to support a "retraction model" first proposed by Marvin and Hohn (48). According to this model the pilus retracts once phage attaches to its tip hence bringing the phage to

the cell surface where penetration occurs (6, 7, 9, 34, 59).

Bacteriophage IKe-Isolation and properties

The filamentous bacteriophage IKe was isolated from sewage using a host strain of Salmonella typhimurium bearing the R plasmid RM98 (38). RM98 does not repress F-associated functions and is by this criterion a Fi^- (F) plasmid. However cells bearing RM98 were unable to propagate the If phages If1 and If2 (38). It was concluded that this plasmid probably represented a separate group in the Fi^- (F) class of R plasmids. This conclusion was supported by the isolation and characterization of IKe which was propagated by RM98 bearing cells, and by the fact that several other strains bearing Fi^- (F) plasmids were insensitive to phages If1 and If2 but propagated IKe (ibid). This group of plasmids was designated N (23). IKe, the N-plasmid specific phage, could not be propagated by strains carrying either ColI or other I-type R plasmids (38).

The physical and chemical properties of the phage have been described (38). It was shown that the phage resembles other filamentous bacteriophages in its morphology, contains single-stranded DNA, no RNA, and has a length distribution similar to that of the If phages (0.9-1.3μ (52)). In addition the phage is relatively heat resistant (a property characteristic of filamentous bacteriophages) and like M13 it is sensitive to the proteinase Nagarase (48, 79).

It was shown however that antiserum prepared against IKe particles does not inactivate either M13 or If phages; IKe is therefore antigenically different from M13 and If (38).

Adsorption of IKe - background and purpose of the present study.

Since both Ff and If phages were found to adsorb to the tips of specific pili, it was of interest to examine the manner in which IKe attaches to its host. For this purpose RM98 the IKe-specific N plasmid was introduced into the F⁻ E. coli K12 strain JE2571. The strain is unable to synthesize either flagella or common pili. On this cell a pilus was therefore expected to be easily detected should RM98 code for the synthesis of such a structure.

No pilus-like structure could be observed by electron microscopy following the introduction of RM98 plasmid into the cell. When either F42 or the derepressed Col I factors were introduced into the plasmidless host strain, attached as well as detached pili were readily observed (R. Iyer in 14).

These findings suggested the possibilities that (1) RM98 transfer is pilus-mediated, and (2) the attachment of IKe, unlike the attachment of all other known plasmid-specific filamentous bacteriophages, does not require a pilus-type receptor.

The conjugal transfer of F- and I-like plasmids to recipient cells and the attachment of the F- and I-specific bacteriophages have been shown to require the same host cell structure - the sex pilus. Thus, a study of the early steps in the infection by IKe appeared essential particulary with respect to the manner of its attachment and possible differences between the attachment of IKe and that of other filamentous bacteriophages.

it could possibly lead to information pertaining to the mechanism of conjugal transfer of RM98, and possibly other naturally occurring plasmids.

Several methods have been shown to be effective in the study of adsorption of filamentous bacteriophages as well as other groups of bacterial viruses. They generally depend on and result in the separation of phage infected (and uninfected) cells from unadsorbed (free) phage following the incubation of phage-host cell (adsorption) mixtures. Each of the separated fractions can then be assayed to determine the extent of phage attachment. The separation can be achieved by one of several methods. (1) low speed centrifugation (2) killing of cells in the mixture (chloroform is widely used) thus allowing the survival of unattached phage only, (3) treatment of the mixtures with phage antiserum or specific proteinases which inactivate free as well as attached and unpenetrated phages (2, 74), (4) filtration of mixtures through filters which are permeable to phage but not to bacteria (12).

Infected cells can be additionally assayed if required in order to ascertain the fraction of the attached phage which penetrated the host cells. In the case of filamentous phages which adsorb to tips of pili, blending or sonication of the infected cell have been effective in removing sex pili and as well as attached (but unpenetrated) phages (34).

In the application of these methods to the study of the attachment of IKe several difficulties were encountered:

(1) IKe was shown to be sensitive to chloroform (36).

(2) Preliminary rxperiments had indicated that IKe particles suspended in a variety of holding media, tended to adsorb to membrane filter discs (pore size 0.45μ) expected to permit their passage (R. Iyer unpublished).

(3) IKe antiserum was found to cause a decrease in the viability of IKe-infected cells and an accurate measurement of infective centers following application of IKe antiserum to adsorption mixtures was therefore not possible (R. Iyer, unpublished; S. Dennison, unpublished).

(4) Blending could not be used as means of measuring either attachment or penetration since a pilus-like structure to which IKe adsorbed had not been demonstrated.

The objective of this study (viz. a systematic examination of the first step in IKe infection) depended on the establishing of a method which would permit the quantitation of IKe attachment.

Two assay methods were found to be applicable. They were subsequently used to determine the conditions under which optimal attachment of the phage to its host cell occurs, and to examine some of the properties associated with the bond(s) involved in the attachment.

CHAPTER 2

MATERIALS AND METHODS

Media

Unless otherwise specified bacteria were grown, phages held and adsorption assayed in L broth and on L agar (43).

Bacterial strains

1. E. coli K12 strain JE2571 F^- pil^- fla^- leu^- thr^- lac^- (Y. Nishimura) bearing the IKe specific R plasmid RM98 (37, 38) was the propagating and host strain in all adsorption experiments. The strain was designated JE2571/98-70. The RM98 - associated markers were Ap^r, Tc^r, RTF^+, IKe^s.

2. JE2571, the corresponding R^- strain served as a control in some experiments.

3. JE2571/F42 was the strain used in adsorption experiments with M13. F42, a $F'lac^+$ factor was obtained from V.N. Iyer.

4. E. coli HfrH (strain K37) was the indicator and propagating strain for M13.

Cultivation of strains

All strains were grown overnight in L broth, diluted 50-fold in the same medium and grown to logarithmic phase by incubation at 37°C in a waterbath shaker (Warner Chilcott Laboratories, 100 strokes/min.). At an optical density of 0.4 units at 650nm (Bausch and Lomb Spectronic 20) cultures were chilled in crushed ice. They were prewarmed to the desired temperature before use.

For overnight cultivation of JE2571/98-70,L broth containing 30 μg/ml Ampicillin was initially used. This often resulted in the production of two types of colonies when log cultures were plated: the characteristic small, smooth colony and a large mucoid variant (see figure 1). The latter was found to be a poor indicator for IKe. This colonial variation was effectively eliminated by using overnight cultures of this strain plated on L agar + 30 μg/ml ampicillin, as inoculam for preparing log cultures. Such overnight cultures were stored at 4°C and used for 7 days only.

Phage

The preparation and purification of IKe stocks as well as assay procedures for the phage, and its properties have been previously described (38).

Phage particles (ρ = 1.286g/ml) obtained from the CsCl gradient were additionally purified on a 5-20% sucrose gradient (R. Iyer, unpublished). Phage particles sedimented in two peaks. 99% of the p.f.u. were collected in the fast sedimenting fraction designated peak A. Unless otherwise indicated peak A particles were used throughout. Peak B constituted the minority, slow sedimenting particles.

Preparation of ^{3}H-labelled IKe and M13

IKe and M13 were labelled with 40 μCi/ml ^{3}H-thymidine (New England Nuclear) using the procedure described earlier (38) with the following modifications.

1. The respective host cultures were incubated at 37°C for 30 minutes

with 4.8 μg/ml of ^{3}H-thymidine prior to addition of the phage. This was followed by incubation at 37°C for 2½ hours, at which time ^{3}H-thymidine (4.8 μg/ml) was added and incubation continued for three hours. After removal of cells by centrifugation, NaCl and aqueous Polyethyleneglycol (MW6000) were added to the supernatant to final concentrations of 0.5M and 4% respectively. This was mixed and held at 4°C overnight. Phage was pelleted by centrifugation for 1½ hours at 8000 r.p.m. 4°C, in a Sorvall SS-1A superspeed angle centrifuge, suspended in 0.05M Tris and purified by a CsCl step gradient centrifugation for 18 hours at 26,500 r.p.m., 5°C, in a Beckman model L3-50 ultra-centrifuge with a SW 50.1 rotor.

Fractions were collected by bottom puncture and aliquots diluted in 0.05M Tris pH 7.4 and assayed for radioactivity by counting in aquasol (NEN) in a Beckman LS-250 Liquid Scintillation system. The infectivity associated with the fractions was assayed by the plaque assay(2). The peaks of radioactivity always coincided with those of infectivity and the corresponding fractions were dialyzed for 24 hours at 4°C against 0.05M Tris pH 7.4 and used for the experiments.

Assays for measuring adsorption

1. Supernatant assay

JE2571/98-70 or JE2571/F42 were grown to a density of approximately 3 x 10^8 v.c./ml (viable cells/ml) chilled and warmed to 32°C just before addition of phage. The number of v.c./ml was generally assayed at this point

Phage-cell mixtures were incubated at 37°C for the desired duration then a 1:50 dilution of the mixture was made with chilled assay media to prevent further adsorption. Samples of diluted mixtures were assayed for total p.f.u./ml and then centrifuged (20 minutes, 4°C, 8,200 r.p.m.) and supernatants assayed for the unadsorbed fraction of input phage.

The assay for total p.f.u./ml was performed to check the extent of phage inactivation or propagation during the incubation period.

2. Pellet assay

Cell-phage mixtures were prepared as described above. Aliquots of mixtures were either layered directly or diluted in chilled homologous media and then layered on 5-10 ml of chilled media containing 10% sucrose (w/v). Separation of unadsorbed phage particles from those attached to cells was obtained by centrifugation of the layered mixture for 25' at 2,800 r.p.m., 4°C, in an IEC International model HN centrifuge with a swinging bucket rotor. Pellets (comprising phage-cell complexes and uninfected cells) were gently resuspended in appropriate chilled media and assayed for infective centers/ml Centrifugation under these conditions yielded pellets which permitted a recovery of approximately 90% of the layered cells.

Both supernatant and pellet assays were often applied to the same adsorption mixture and in somecases only one of the methods was used.

Note:

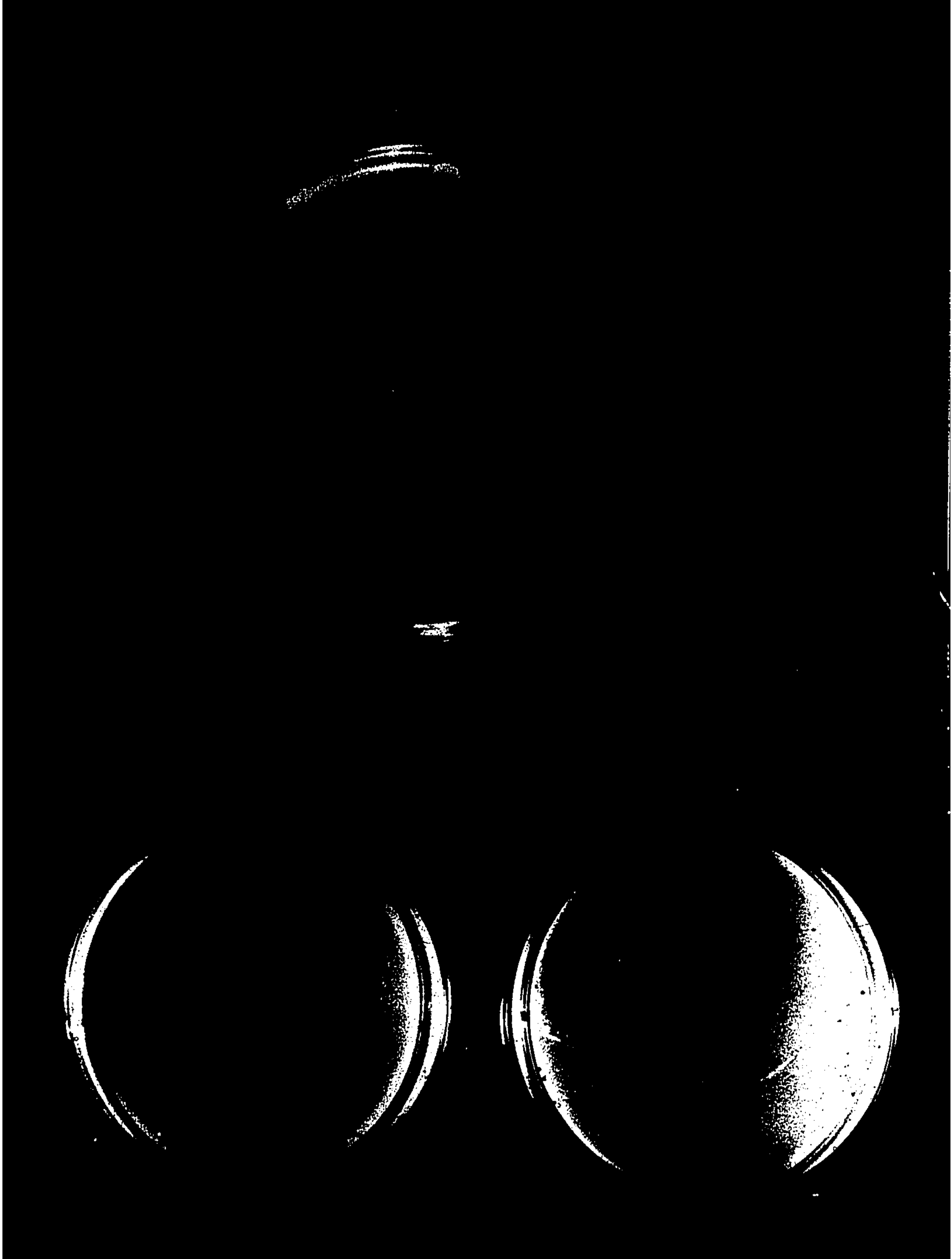
Plaques obtained from the plating of supernatants (unadsorbed

Figure 1: Two types of colonies from a log culture of E. coli JE2571/98-70.

Figure 2: Plaque types obtained by the supernatant and pellet assay methods.

A. Supernatant assay

B. Pellet assay



phage) were usually small and rather turbid. Plating of phage-cell complexes (pellets assays following sucrose centrifugation) however yielded larger and clearer plaques (see Figure 2). This phenomenon is typical of infection by filamentous single-stranded DNA phages and has been previously reported (48).

Penetration assay

Purified phage-cell complexes obtained by the pellet assay described above were suspended in a phosphate buffer (4.0g $(NH4)_2$ HPO_4 and 2.3g $NH_4H_2PO_4$ in 1 liter distilled H_2O (79)) containing 10mg/ml Nagarase (Nagase & Co., Osaka, Japan). Incubation was at 37°C for 15'. Nagarase was removed by rejecting supernatants following centrifugation and pellets were resuspended and assayed for i.c./ml. (infective centers/ml).

This was inferred to yield a measure of the penetrated phage since Nagarase is known to inactivate unpenetrated phage (79) and IKe is sensitive to this enzyme (38).

Other procedures

1. Blending:

40 ml aliquots of the bacterial culture were blended in a 250ml polypropylene bottle held in crushed ice. Blending was for two minutes at 10,000 r.p.m. using a Sorvall Omnimixer Homogenizer.

2. Filtration:

Cell cultures or phage preparations were filtered on sterilized 25 or 47mm Millipore filters discs, type HA, 0.45μ pore size.

CHAPTER 3

RESULTS AND DISCUSSION

A. STABILITY OF THE PHAGE

The stability of IKe in the holding media listed below was tested. Phage was held in the various media at 4°C for a period of nine months. The number of p.f.u./ml was assayed several times during this period.

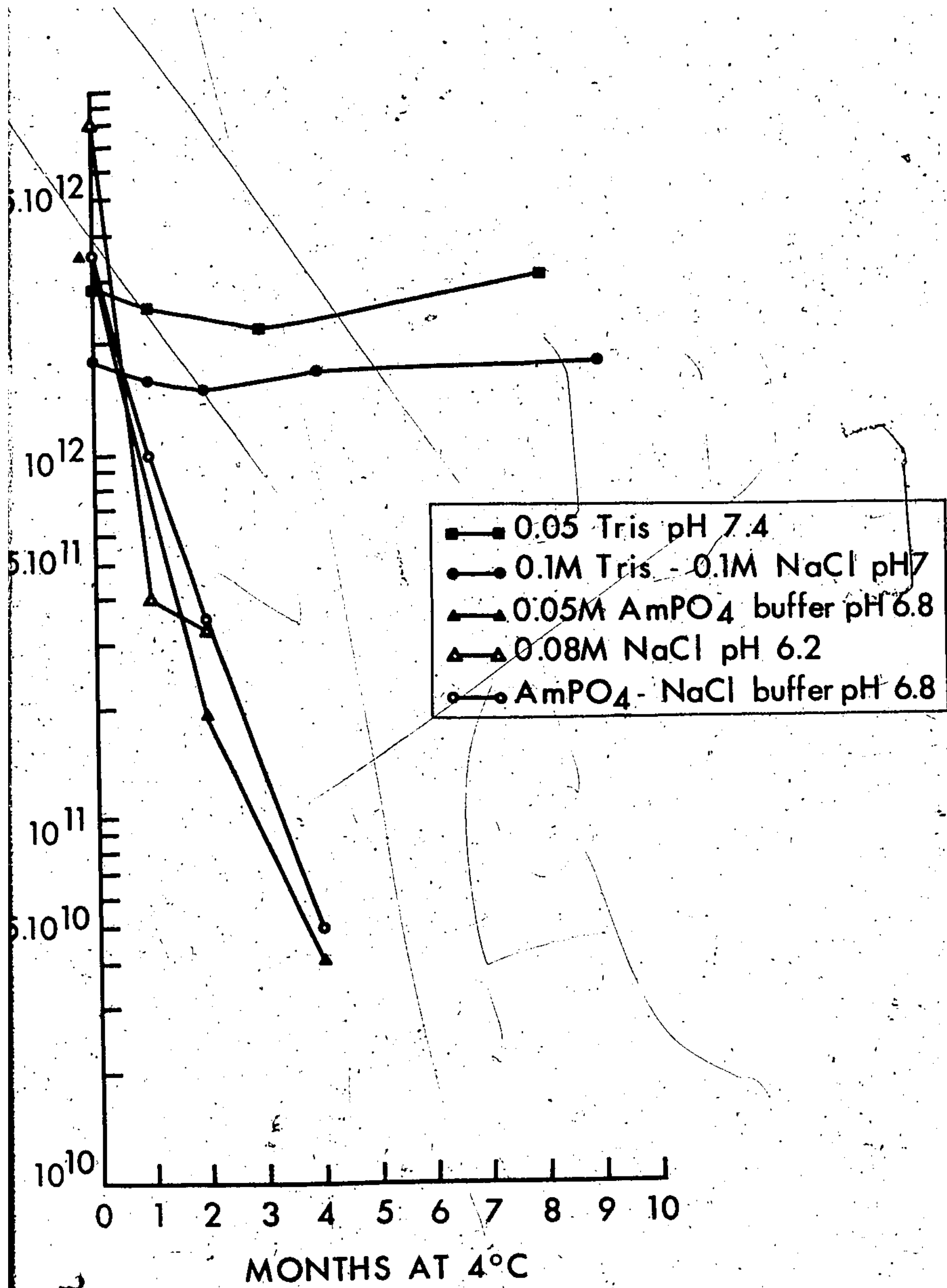
A decrease in the number of infective particles was observed in all media except in 0.05M Tris pH 7.4, 0.1M Tris - 0.1M NaCl pH 7 and L broth (see Figure 3).

When phage was held at 37°C for 1 hour, however, losses in the infectivity of the phage were observed both in 0.05M Tris and 0.1M Tris - 0.1M NaCl. No similar losses could be detected under these conditions in L broth which was therefore routinely used as the holding medium for the phage.

Holding media

1. 0.1M NaCl pH 6.2
2. 0.1M NaCl with various concentrations of $CaCl_2$ (0.001M to 0.1M)
3. 0.05 ammonium phosphate buffer pH 6.8 (79)
4. 0.08M NaCl + 0.05M ammonium phosphate buffer pH 6.8 (79)
5. 0.05M Tris (hydroxymethyl) aminomethane pH 7.4
6. 0.1M Tris - 0.1M NaCl pH 7 (34)
7. M9 (1g NH_4Cl, 3g KH_2PO_4, 6g Na_2HPO_4, in 1 liter distilled H_2O) (2).
8. L broth (43).

Figure 3. The stability of IKe in various holding media.



5.10^{12}
10^{12}
5.10^{11}
10^{11}
5.10^{10}
10^{10}
0 1 2 3 4 5 6 7 8 9 10
MONTHS AT 4°C
0.05 Tris pH 7.4
0.1M Tris - 0.1M NaCl pH7
0.05M $AmPO_4$ buffer pH 6.8
0.08M NaCl pH 6.2
$AmPO_4$ - NaCl buffer pH 6.8

B. THE ADSORPTION OF IKe

1. Effect of phage concentration

Using a cell density of approximately 3×10^8 v.c./ml, measurable losses from supernatants due to adsorption were obtained only if phage concentrations of approximately 7×10^5 p.f.u./ml or lower were used. Adsorption as measured by the supernatant assay, was therefore examined at a m.o.i. of 0.002 or less. In certain experiments, however, where adsorption had to be studied at higher m.o.i. (see table 10) the pellet assay was used instead. At m.o.i. ranging from 0.3 to 30, when adsorption mixture was incubated at 37°C for 15', 5-0.5% of input phage respectively could be recovered as infective centers by this method.

2. Effect of cell concentration

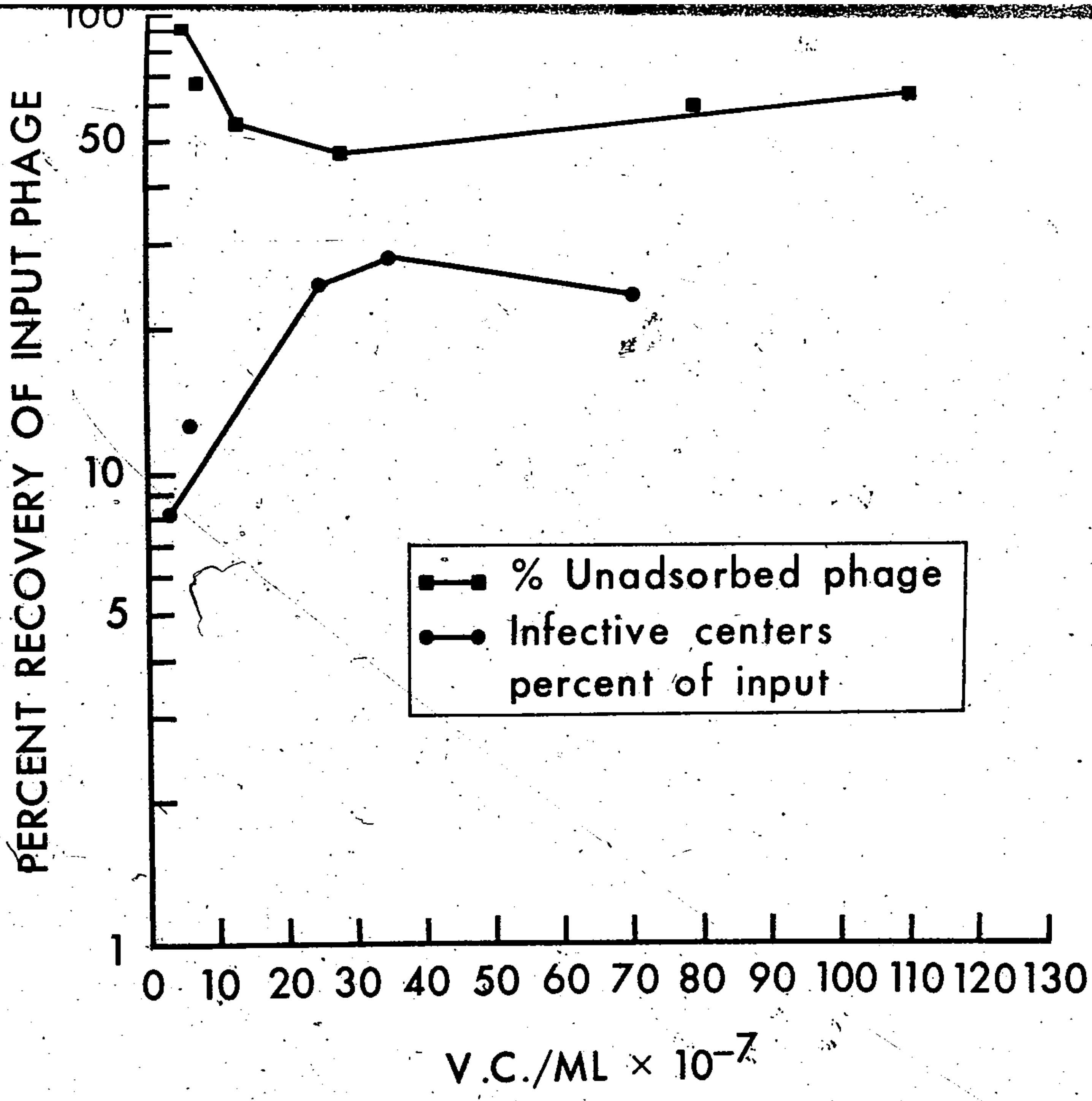
The dependence of the rate of adsorption on the concentration of viable cells present in the adsorption mixture was examined. Both unadsorbed and attached phage were assayed in mixtures containing an increasing number of viable host cells.

The results shown in Figure 4 indicate that at low cell concentrations (up to approximately 4×10^8 v.c./ml under the conditions of the experiment) the fraction of phage that adsorbs to host cells increases linearly with the increase in number of host cells. At higher cell concentrations, however, the number of attached phage levels off and even decreases slightly.

These results are similar to results reported with T4 (75, 84)

Figure 4. Effect of host cell concentration on adsorption.

Legend: JE2571/98-70 cells were harvested by centrifugation and pellets resuspended in chilled L broth to give cell concentrations ranging from 1×10^7 to 1×10^9 v.c./ml. Cells were warmed to 32°C and infected with 5×10^5 p.f.u./ml of IKe. Incubation was for 10' at 37°C. Supernatant and pellet assays were done on separate occasions.


PERCENT RECOVERY OF INPUT PHAGE
100
50
10
5
1
0
10
20
30
40
50
60
70
80
90
100
110
120
130
V.C./ML × 10⁻⁷
% Unadsorbed phage
Infective centers
percent of input

and ϕX174 (26) but differ from the results obtained with M13 (79). They suggest that at high cell concentrations diffusion is no longer the only factor limiting the rate of adsorption.

3. k - The attachment rate constant

a. Peak A: The rate of phage adsorption has been shown to follow first order kinetics in respect to phage concentration (26, 74, 78). Since adsorption of IKe has been shown to increase linearly as cell concentration increases (see figure 4) within a certain range of cell concentrations, the rate constant for the reaction in that range can be determined by the equation first postulated by Schlesinger in 1934 (72) to describe the kinetics of the reaction between phages and their host cells.

$$-\frac{dP}{dt} = kPC$$

where:

P = concentration of unattached phage at time t.

C = concentration of host cell

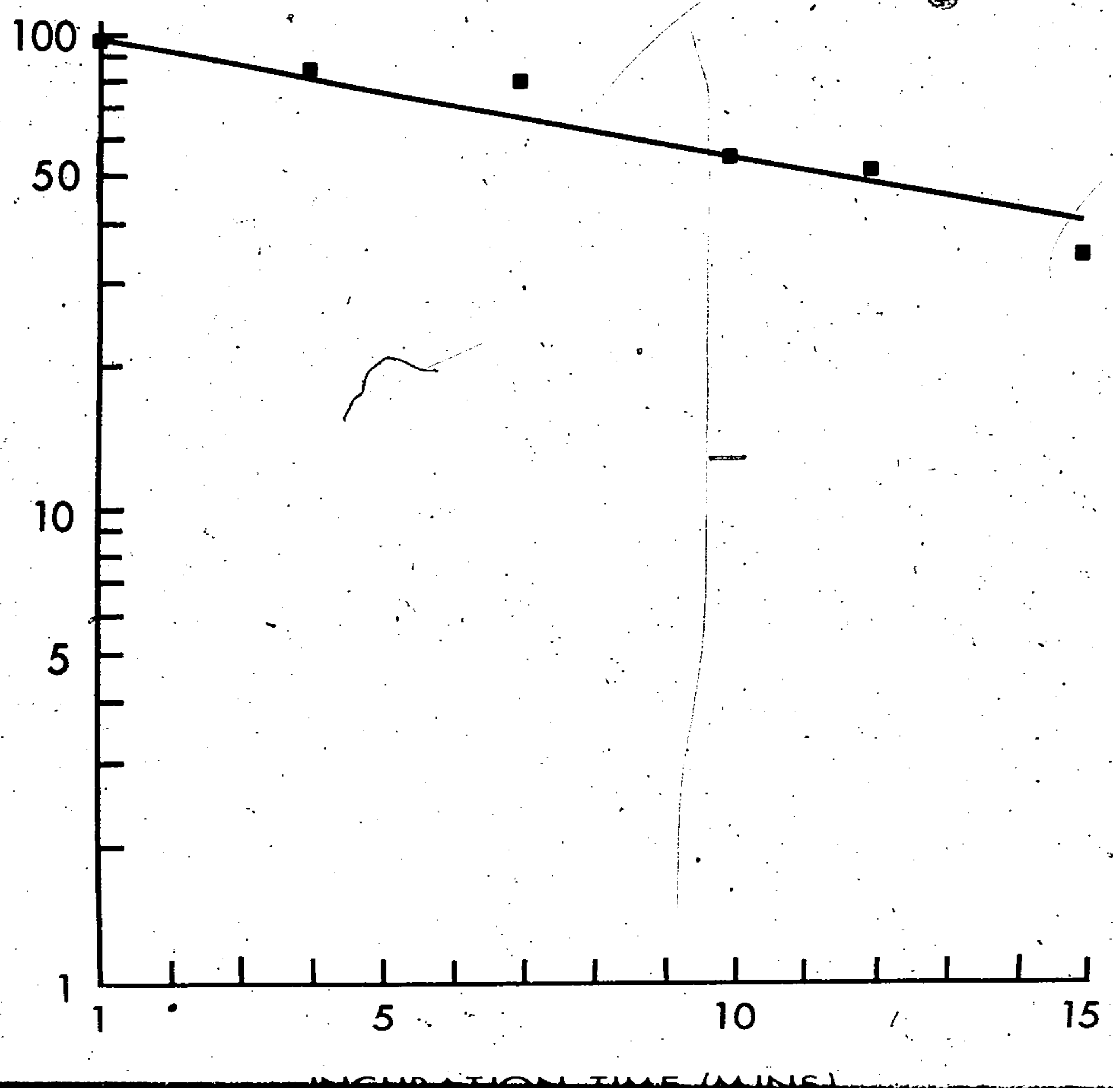
k = rate constant.

The integration of this differential would be $\log \frac{P_1}{P_0} = \frac{-1}{2.3} kCt$ and k can be calculated from the slope of the curve describing the above relationship $k = \frac{2.3}{C(t_1 - t_0)} \log \frac{P_0}{P_1}$

The rate constant for the attachment of IKe peak A was calculated using the slope of the curve presented in Figure 5.

The cell concentration was 2.9×10^8 v.c./ml; phage concentration

Figure 5. The adsorption of IKe (peak A) in L broth.



PERCENT UNADSORBED PHAGE
100
50
10
5
1
1
5
10
15

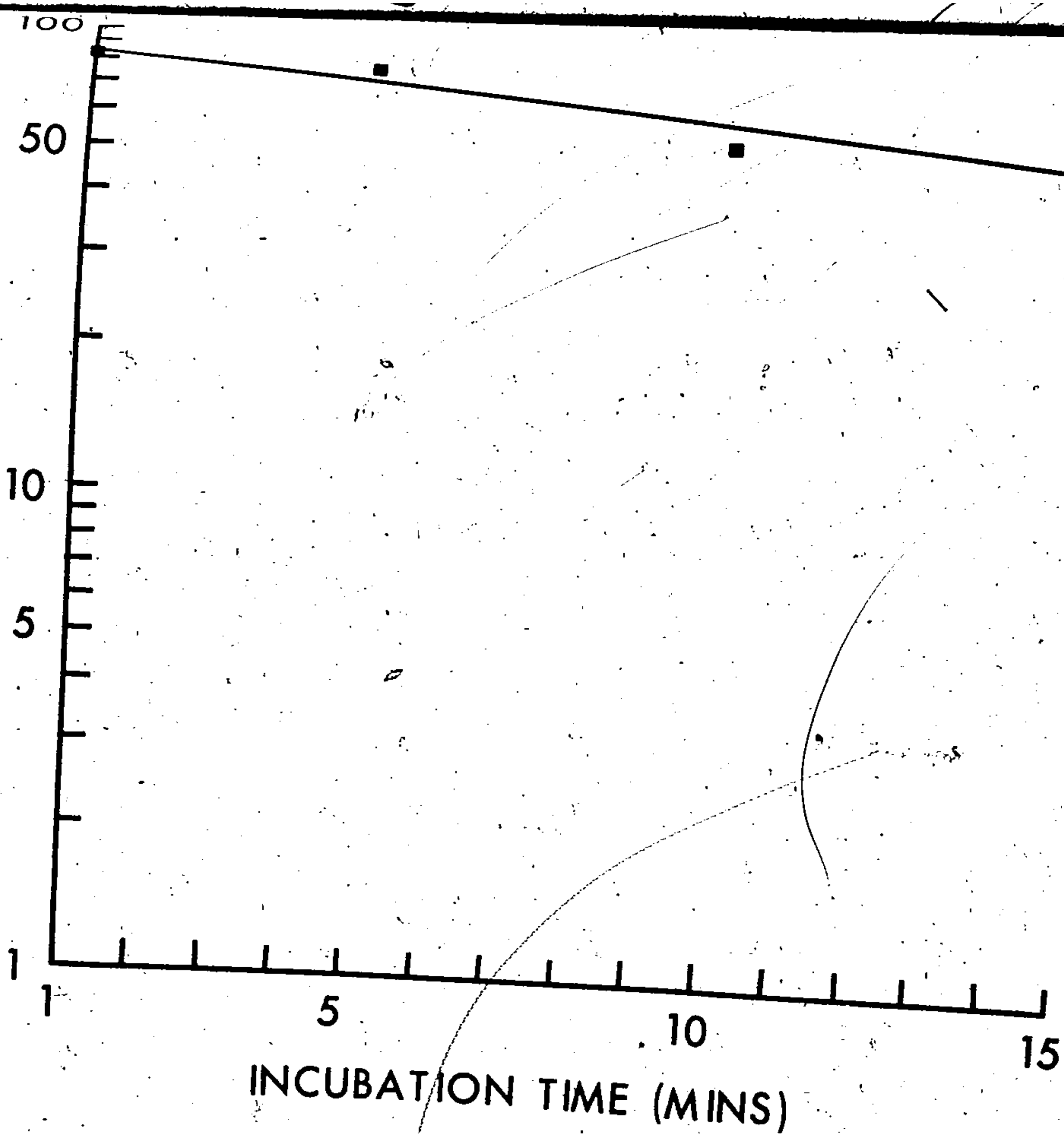

PERCENT UNADSORBED PHAGE
100
50
10
5
1
1
5
10
15
INCUBATION TIME (MINS)

Figure 6. The adsorption of peak B particles in L broth.

was 6×10^4 p.f.u./ml and unadsorbed phage was measured by the supernatant method. k calculated from the slope was 23×10^{-11} $Cm^3 min^{-1}$. Similar values were obtained using cell concentrations of up to 4×10^8 v.c./ml.

This value is considerably lower than that obtained in the case of some of the T phages (values between 200 and 450×10^{-11} cm^3 min^{-1} were obtained for T1, T2, T3 and T4) (66) but higher than that reported for M13 under similar conditions (3×10^{-11} cm^3 min^{-1}) (79).

b. Peak B: The rate constant for the adsorption of peak B particles (see Materials and Methods page 14) was calculated in the same manner (see Figure 6).

Cell and phage concentrations were 4.1×10^8 v.c./ml and 7.5×10^4 p.f.u./ml respectively. k for peak B particles was 7.6×10^{-11} cm^3 min^{-1}. This is 66% lower than that obtained for peak A particles.

4. Effect of the ionic concentration of the medium

a. NaCl: Attachment of TKe in aqueous solutions containing increasing concentrations of NaCl was assayed both by the supernatant and pellet methods on separate occasions.

Results are shown in Figure 7. Using either assay method adsorption was shown to be maximal at a concentration of 0.1M NaCl. Phage T2 as well as M13 have also been shown to require this concentration of NaCl for maximum adsorption (28, 79).

The results indicate however that whereas 30% of the input

phage could not be recovered in the supernatant following adsorption at the optimal NaCl concentration, only 1.9% of the input phage could be recovered as infective centers (pellet assay). This discrepancy was noted at all NaCl concentrations used.

An assay of the number of p.f.u./ml in adsorption mixtures, before and after incubation at 37°C, indicated no phage inactivation at the salt concentrations used. It was observed, however, that phage particles suspended in 0.1M NaCl loose about 50% of their plaque forming ability when incubated _per se_ (without cells) at 37°C for 30 minutes.

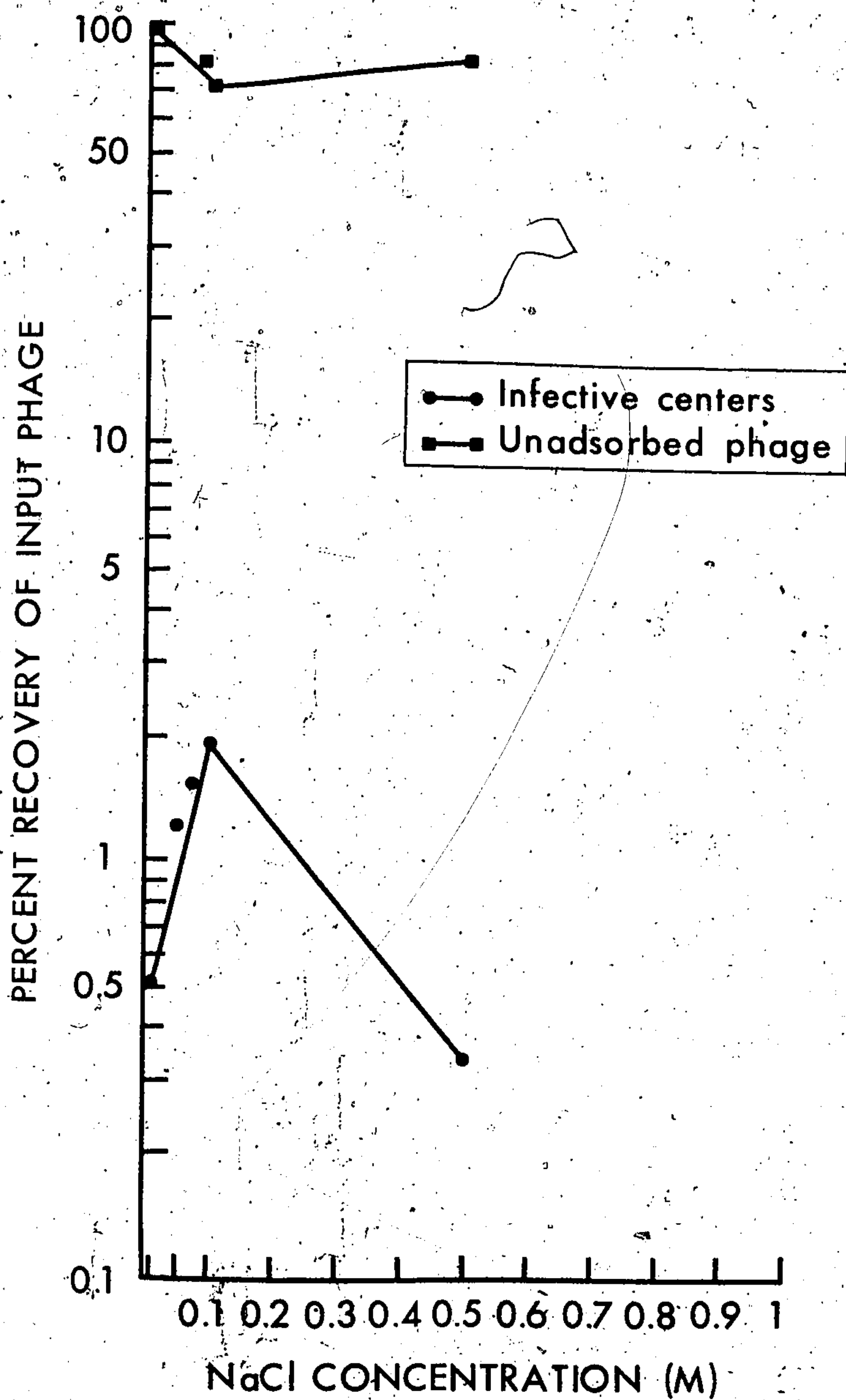
b. The addition of $CaCl_2$: Adsorption was measured next in 0.1M NaCl supplemented with various concentrations of $CaCl_2$ since divalent cations have been shown to be necessary for the attachment of other phages (24, 26). Results are shown in Figure 8. The addition of $CaCl_2$ at all the concentration tested did not result in increased adsorption. These findings suggest that the presence of divalent cations in the media is not required for the adsorption of phage IKe.

It should be noted also that the fraction of free phage in supernatants increased as incubation at 37°C proceeded, in 3 out of 4 media tested. The significance of these increases remains unclear at the present time.

In a separate experiment it was demonstrated that the stability of IKe particles held in 0.1M NaCl is increased by the addition of 0.002M $CaCl_2$ to the medium. Consequently in subsequent experiments calling for

Figure 7. The adsorption of IKe in aqueous solutions of NaCl.

Legend: Aliquots of the host cell culture were harvested by filtration washed with and resuspended in chilled aqueous solutions of NaCl. Cell suspensions were infected with 6×10^4 p.f.u./ml of IKe. Incubation at 37°C was for 30 minutes. Unadsorbed phage and infective centers were assayed on separate occasions. Cell concentration was 1.4×10^8 v.c./ml in supernatant assays and 4×10^8 v.c./ml in pellet assays.


PERCENT RECOVERY OF INPUT PHAGE
100
50
10
5
1
0.5
0.1
Infective centers
Unadsorbed phage
0.1 0.2 0.3 0.4 0.5 0.6 0.7 0.8 0.9 1
NaCl CONCENTRATION (M)

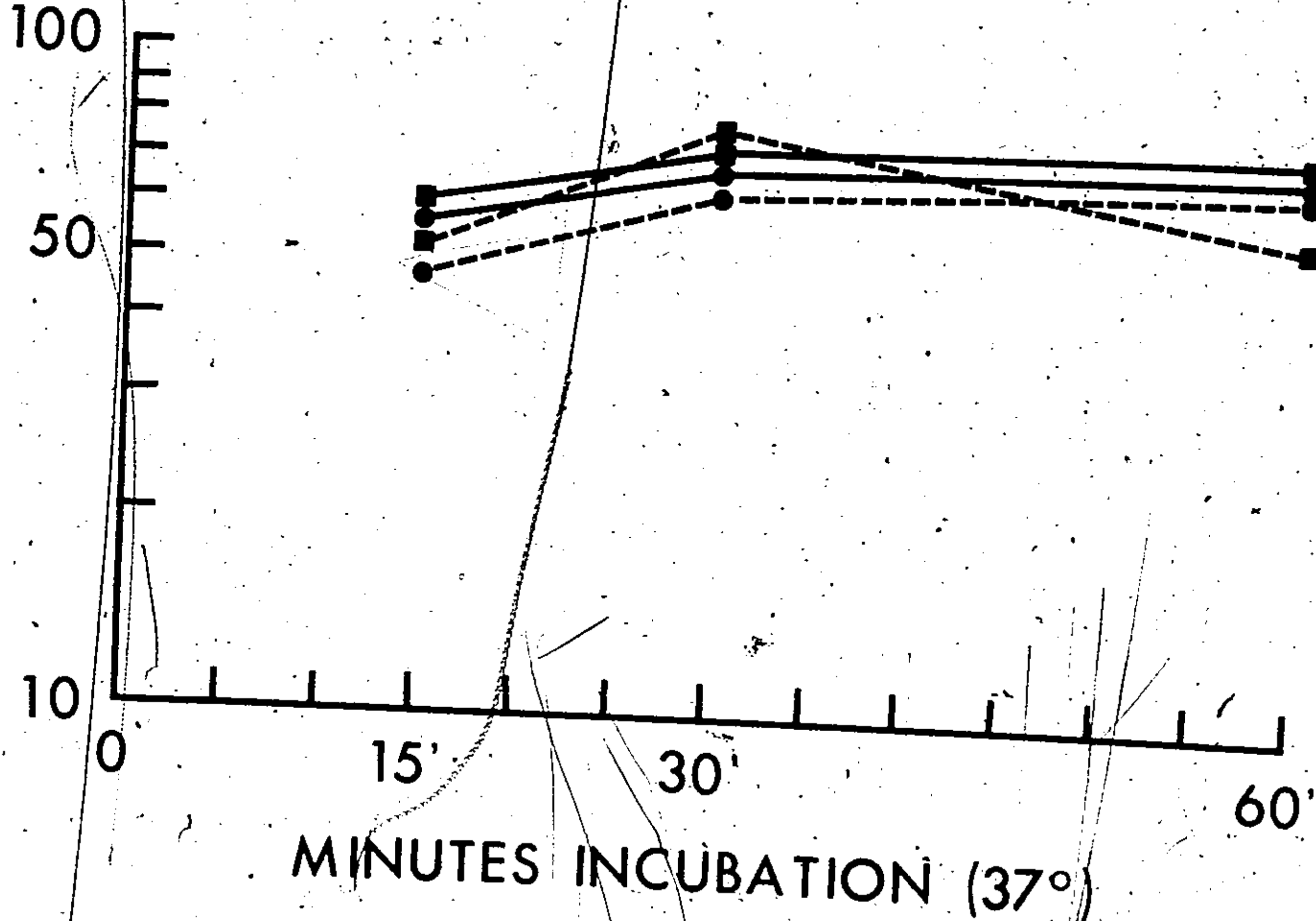

Adsorption in 0.1M NaCl
Adsorption in 0.1M NaCl + 0.1M CaCl2
Adsorption in 0.1M NaCl + 0.01M CaCl2
Adsorption in 0.1M NaCl + 0.001M CaCl2
PERCENT
UNADSORBED PHAGE
100
50
10
0
15'
30'
60'
MINUTES INCUBATION (37°)

Figure 8. Adsorption of IKe in 0.1M NaCl + $CaCl_2$.

Legend: Host cells were suspended in indicated media as described in the legend to figure 7. IKe held in chilled 0.1M NaCl was added to all suspensions to a final concentration of 7×10^4 p.f.u./ml. Incubation was at $37^{\circ}C$ and supernatants were assayed after 15, 30 and 60 minutes of incubation.

the use of a defined medium, the basal medium (0.1M NaCl) was supplemented with 0.002M $CaCl_2$ with additional supplements added as required (see table [illegible]). A similar stabilization of T5 particles held in 0.1M NaCl by the addition of 10^{-5} M divalent cations has been reported (1, 40).

[illegible]. Adsorption in NaCl-free L broth: In a further attempt to examine the extent to which adsorption is dependent on the presence of NaCl, adsorption in L broth (containing 0.1M NaCl) and in a NaCl-free control were compared. The results are shown in Figure 9. They demonstrate that in the absence of NaCl adsorption is significantly reduced even in a nutrient medium.

It can therefore be concluded that adsorption of IKe to its host requires a univalent salt—NaCl and is optimal at a concentration of 0.1M.

c. Attachment of IKe to filters: Garen and Puck reported in 1951 that phages T1 and T4 can adsorb to glass filters. This adsorption was similar to the adsorption to host cells in its dependence on the ionic constitution of phage holding media (66, 67).

In preliminary experiments with IKe (R. Iyer, unpublished) it was observed that phage particles when filtered through membrane filter discs (Millipore) could under certain conditions neither be recovered in the filtrate nor be eluted by suspension of filters in various media or different salt solutions. This observation was investigated further in light of the fact that (1) phage attachment to host cells occurred only

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c. Adsorption in NaCl-free-L broth: In a further attempt to examine the extent to which adsorption is dependent on the presence of NaCl, adsorption in L broth (containing 0.1M NaCl) and in a NaCl-free control were compared. The results are shown in Figure 9. They demonstrate that in the absence of NaCl adsorption is significantly reduced even in a nutrient medium.

It can therefore be concluded that adsorption of IKe to its host requires a univalent salt-NaCl and is optimal at a concentration of 0.1M.

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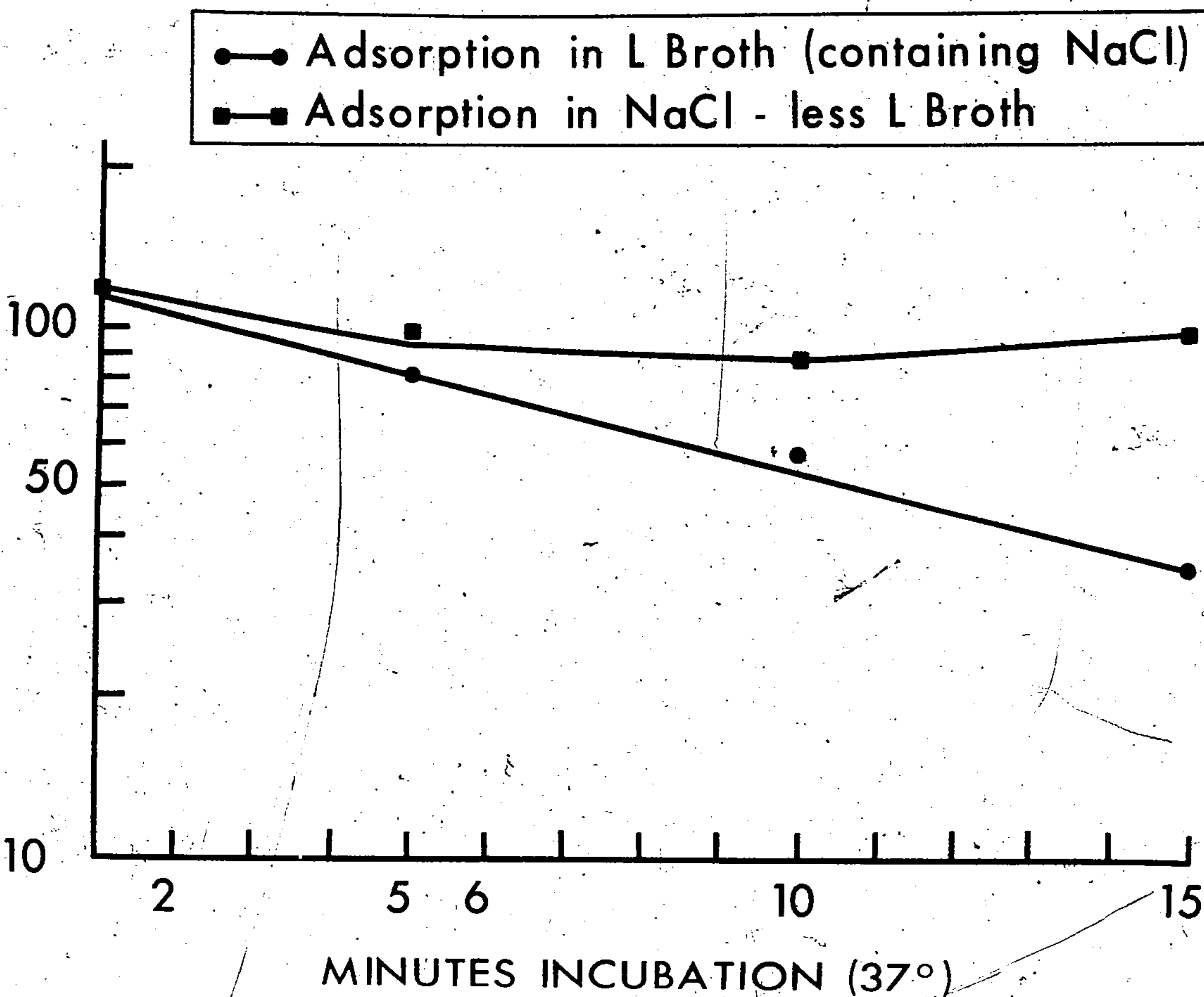
Figure 9. Effect of NaCl on IKe adsorption in L broth.

legend: 2 aliquots of a log culture of the host cells were filtered, washed and resuspended in chilled L broth either containing or lacking 0.1M NaCl. Cell density was 3×10^8 v.c./ml. Phage was added as described in the legend to figure 8 and incubation was at 37°C for 15'.


Adsorption in L Broth (containing NaCl)
Adsorption in NaCl - less L Broth
PERCENT UNADSORBED PHAGE
100
50
10
2
5
6
10
15
MINUTES INCUBATION (37°)

in the presence of a specific salt concentration and (2) attachment of some of the T phages to filters had been reported (above).

The results presented in Table 1 indicate that IKe particles held in L broth and layered on membrane filters can easily be eluted from the filters when the latter are washed with distilled (deionized) H_2O. Only a negligible portion of the phage can be eluted however, when filters are washed with 0.08M NaCl.

Thus, attachment of IKe to both host cells (see figure 7 and figure 9) and the membrane filters (mixed esters of cellulose) requires a specific and similar concentration of NaCl in attachment media. As in the case of T phages (78), this may suggest that the primary reaction between IKe and its host is ionic in nature. Hence, it is mediated by attracting electrostatic charges present on both surfaces rather than the activity of a specific biological entity present on the surface of host cells (e.g. enzyme).

It is assumed that the role of the ionic environment is to neutralize sufficient repulsing electrostatic charges on the surfaces of host cell and phage thus enabling attachment (65, 66, 79). The fact that both host cells and filter discs require the same concentration of NaCl for adsorption may therefore be due to a similar net charge on both surfaces.

Aside from the theoretical implications, the information presented in Table 1 may be used in devising a method for sterilization

Table 1: Elution of IKe from filter discs.

Eluants	fraction of IKe eluted (% of input)
distilled deionized H_2O	51
0.01M NaCl	0.18
0.08M NaCl	0.00024
L broth	0.24

Legend: 0.1 ml of an IKe (band I (38)) suspension in L broth containing 8.2×10^8 p.f.u./ml was filtered through Millipore filter discs and washed with 10 ml of each of the eluants. Filtrates were assayed for p.f.u./ml.

of IKe suspensions.

5. Effect of Temperature

a. The optimal temperature: The attachment of IKe to its host cell is temperature dependent as shown in Figure 10. There is very little or practically no attachment at 0°C followed by a 10-fold increase in the number of attached particles at 30°C. Adsorption levels off at this temperature and decreases considerably at 45°C. Control experiments have shown no reduction in the number of viable cells or p.f.u. due to incubation at 45°C.

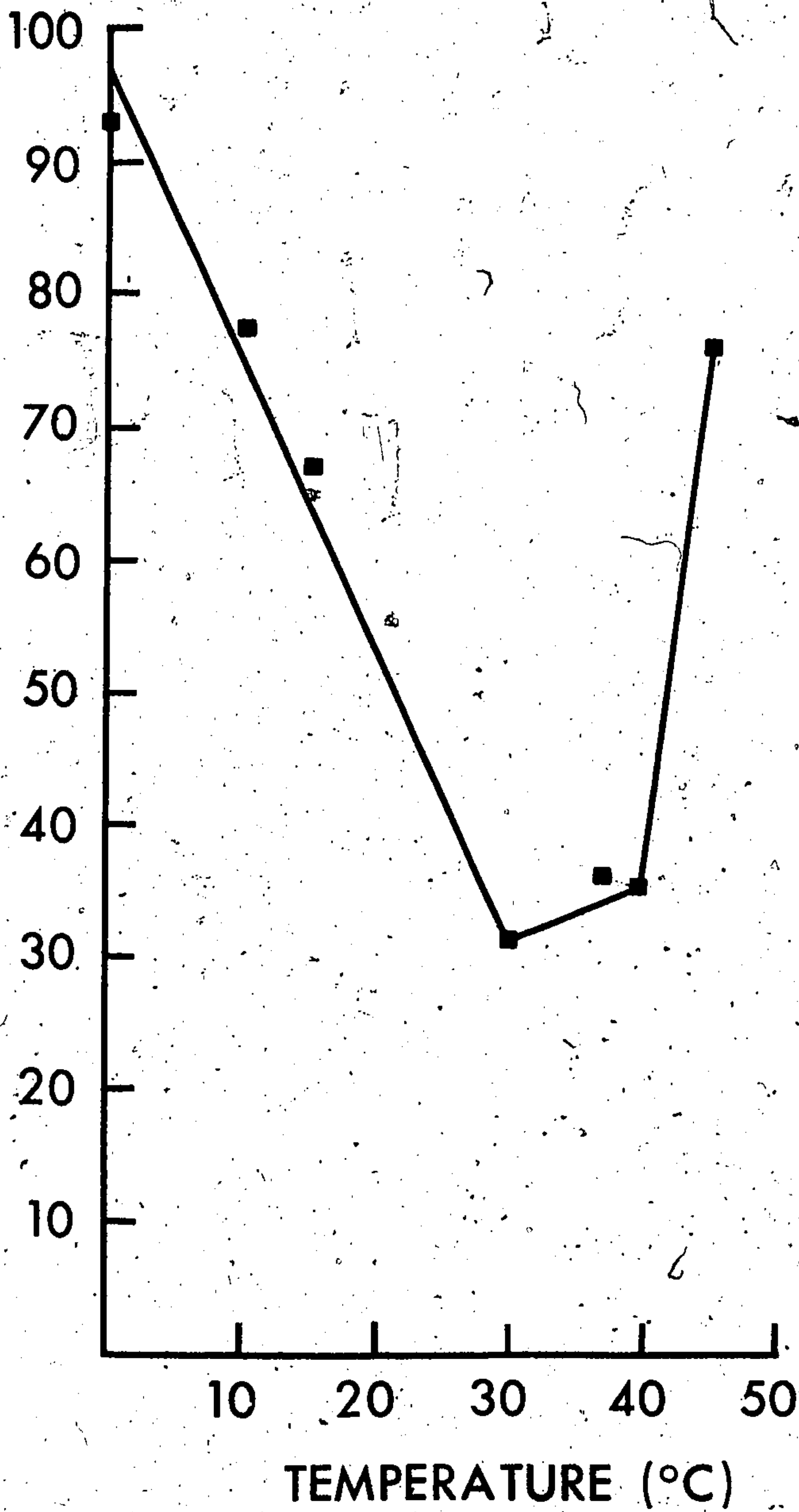
b. Adsorption at 0°C: Adsorption at 0°C was assayed on several occasions both by the supernatant and pellet methods. In addition pellets obtained by direct centrifugation (see supernatant assay page 15) were also assayed for infective centers. Results of two experiments are shown in Figure 11. Figures obtained at different m.o.i. and incubation periods (up to 2 hours) did not vary considerably. They suggest that very little or no adsorption occurred at 0°C.

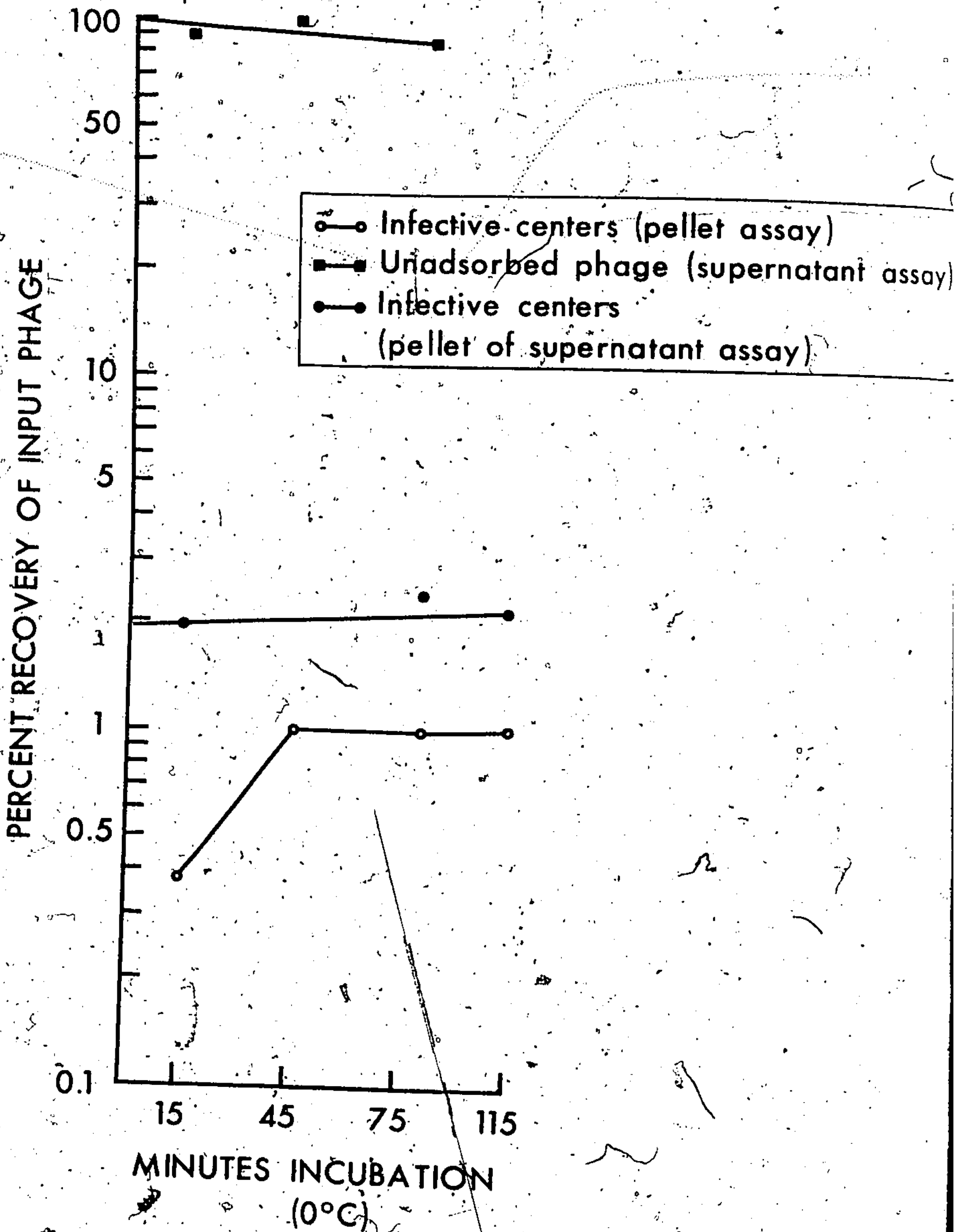
Thus, when required, adsorption of IKe to its host cells can be effectively stopped by a rapid chilling of adsorption mixtures to 0°C.

c. Preincubation of host cells at 45°C: The nature of the reduction in the adsorption of IKe shown to occur at 45°C (see Figure 8) was examined. Host cells were incubated at 45°C prior to addition of IKe, and incubation of mixtures was at 32°C. Results are shown in Table 2. Supernatants and pellets were assayed on separate occasions.

Figure 10. Effect of temperature on adsorption.

Legend: 8 chilled aliquots of the host culture (2×10^8 v.c./ml) were prewarmed to the desired temperature prior to addition of 7×10^4 p.f.u./ml of IKe. Incubation was at the indicated temperature for 20'. Adsorption mixtures were chilled and centrifuged and supernatants were assayed.


100
90
80
70
60
50
40
30
20
10
PERCENT UNADSORBEB PHAGE
10
20
30
40
50
TEMPERATURE (°C)



100
50
10
5
1
0.5
0.1
PERCENT RECOVERY OF INPUT PHAGE
Infective centers (pellet assay)
Unadsorbed phage (supernatant assay)
Infective centers
(pellet of supernatant assay)
15
45
75
115
MINUTES INCUBATION
(0°C)

Figure 11. Adsorption at 0°C.

Legend: Cell density was 3×10^{8} v.c./ml. Phage concentration was 6×10^{4} p.f.u./ml. Samples withdrawn from adsorption mixtures at indicated times were centrifuged without prior dilution. Both the supernatants and the pellets obtained by this centrifugation were assayed. Additionally samples from adsorption mixtures were simultaneously assayed by the pellet assay.

Table 2. Effect of preheating of host cells to 45°C on the adsorption of IKe

	Preincubation of cells at 32°C	Preincubation of cells at 45°C	The reduction in adsorption*
Unadsorbed phage[a] (% of input)	35	75	62%
Infective centers[b] (% of input)	15	1.7	89%

* due to preincubation of cells at 45°C.

Legend: a. Host cells (1×10^8 v.c./ml) were incubated at either 32°C or 45°C for 25' prior to addition of phage (5×10^4 p.f.u./ml). Incubation of both adsorption mixtures was for 15' at 32°C; unadsorbed particles were assayed by the supernatant method.

b. Phage cell mixtures were prepared as above (a). Cell density was 2×10^8 v.c./ml and phage concentration 6×10^4 p.f.u./ml. Incubation at 32°C was for 12'. Infective centers were assayed by the pellet method.

Both supernatants and pellets showed a reduction in the adsorption following preincubation of host cells at 45°C. The reduction could be due to: (1) denaturation of the adsorption site (2) inactivation of a post-adsorption temperature-sensitive step, necessary for the formation of stable phage-cell complexes (3) both 1 and 2.

The recovery of a large portion of input phage as unadsorbed particles in the supernatant seems to support the first possibility. However, the fact that a higher relative reduction in adsorption is registered by the pellet assay (see Table 2) may be interpreted as evidence supporting the third possibility.

Inactivation of the receptors for M13 due to incubation of its host cells at 45°C was also reported (79).

6. Effect of pH

a. The optimal pH: The adsorption of IKe as a function of pH was examined. Supernatant and/or pellet assays were used. The effect of pH was studied in either L broth or NCGLT (see legend to Table 6) since optimal adsorption has been observed in these media. Control experiments were simultaneously set up to measure changes in the plaque forming ability of the phage *per se* under the experimental conditions i.e. (a) incubation at 37°C at the various pHs used; (b) centrifugation following dilution in the respective media. This was found necessary in view of the observation that phages aggregate at low pH (68).

Results are summarized in Table 3. They indicate the following:

(1) There was apparently no significant difference in the extent of adsorption at either pH5.0 or 7.3 and the phage was stable under these conditions.

(2) The number of phage particles recovered in the supernatant at pH4.2 was considerably lower than that recovered at pH5.0 or 7.3. This was not due to inactivation of phage as indicated by the results of control I (line 1) where 100% of input phage particles were recovered (Table 3, Col. 2). It could, however, be partially due to effect of centrifugation at this pH, as indicated by control II (line 1) where only 75% of input phage were recovered (Table 3, Col. 3).

(3) Incubation of phage particles at pH9.8, 37°C, caused a loss of 50% in the plaque forming ability of the phage. The loss was augmented by centrifugation (Table 3, Col. 2, 3). It is therefore difficult to determine whether the loss of 18% of input phage from supernatant of the adsorption mixture at this pH (Table 3, Col. 4) was due to phage inactivation or to adsorption.

The measurement of infective centers formed at pH9.8 was made impossible by the fact that close to 90% of the cells lost their viability at this pH.

b. <u>Adsorption at pH4.0:</u> That non-specific attachment between phage T2 and its host occurs at low pH has been reported (68). The possibility that a similar type of reaction between IKe and its host is responsible

Table 3. Effect of pH on the adsorption of IKe.

	Phage stability (p.f.u./ml recovered) at 37°C, 10' Control I	Control II	Phage adsorption
pH of L broth	before centrifugation (% of input)	after centrifugation (% of input)	free phage in supernatant (% of input)
4.2	100	75	18
5.0	100	100	63
7.3	100	100	71
9.8	50	25	82

Legend: Cells (3×10^8 v.c./ml) were harvested by centrifugation and resuspended in chilled L broth at the desired pH. Cells were warmed to 32°C and phage added (6×10^4 p.f.u./ml). Incubation was for 10' at 37°C and supernatants were assayed for unadsorbed phage (Col. 4). Col. 2 represents recovery of phage following incubation at the indicated pH. Col. 3 describes the effect of centrifugation on recovery of phage following incubation.

for the high losses of p.f.u. from supernatants at pH 4.2 (see Table 3, Col. 4) was next examined. Two experiments were set up.

In the first experiment (Table 4) both supernatants and pellets from adsorption mixtures at pH4.0 or 7.0 were assayed. By comparing the results obtained it could be determined whether or not the p.f.u. lost from supernatant at pH4.0 are specifically attached to hosts, and cause a corresponding increase in the number of infective centers in the pellet.

The results shown in Table 4 indicate that while the loss of free phage from supernatant at pH4.0 was 21-fold higher than that at pH7.0, this was not correspondingly reflected in an increased number of infective centers. In fact the recovery of infective centers at pH4.0 was 30% lower than that at pH7.0 (Table 4, Col. 3). Control assays indicated no phage inactivation under the experimental conditions (legend to table 4). These findings suggest that a large portion of the particles lost to the pellet at pH4.0 did not cause an infection of the bacterial cell.

In a second experiment IKe particles were incubated at pH4.0, 37°C, with either JE2571 (R^-) or JE2571/98-70. Supernatants of both mixtures were assayed for unadsorbed phage.

The results shown in Table 5 indicate that the increased loss from supernatant at pH4.0 was not specific to JE2571/98-70 since it was observed with the R^- strain as well.

In light of the data obtained in all three experiments (Tables

Table 4. Adsorption of IKe at pH4.0 and 7.0.

pH	Free phage in supernatant (% of input)	Infective centers (% of input)
4.0	2.4	19
7.0	51	27

Legend: Aliquots of host culture were filtered and filters resuspended in chilled NCGLT at pH 4.0 or 7.0. Suspensions contained 3×10^8 and 4.5×10^8 v.c./ml respectively. Phage held in NCGLT was added to each suspension to a final concentration of 6×10^4 p.f.u./ml and incubation was at 37°C for 20'.

Table 5. The specificity of IKe adsorption at pH4.0.

Culture	Free phage in supernatant (% of input)
JE2571/98-70	3%
JE2571 (R^-)	2.1%

Legend: JE2571 and JE2571/98-70 were grown to a concentration of 3×10^8 v.c./ml. Aliquots were centrifuged and pellets suspended in chilled L broth at pH4.0. To the cells (warmed to 32°C), 6×10^4 p.f.u./ml of IKe were added. Phage cell mixtures were incubated for 15' at 37°C and unadsorbed phage assayed.

3, 4, 5) two possible explanations for the low yield of p.f.u. in supernatants at pH4.0 or 4.2 can be considered.

1. Phage aggregation resulting in increased sedimentability of particles *per se* at the low pH, this sedimentability being aided by cells if present in the low pH medium (i.e. cells acting as carriers).

2. A non-specific attachment of phage to cells occurs at these pHs.

The possibility that cells merely serve as carriers thus enabling the sedimentation of aggregated phage particles was eliminated. The addition of JE2571/98-70 cells to a tube containing a chilled suspension of IKe at pH4.0 just before centrifugation did not cause a greater number of particles to sediment and the yield of particles in the supernatant was not lower than that obtained in the absence of cells (75% of input, Table 3, Col. 3).

That aggregation of phage is partially responsible for the low recovery of particles in supernatant is indicated by the results shown in Table 3, Col. 3. Incubation of the phage with host cells at 37°C prior to centrifugation, however, significantly decreased the number of particles recovered in the supernatant (Table 3, Col. 3 and 4).

In view of the findings described above, the interpretation that a non-specific attachment between IKe and its host takes place at pH4.0-4.2 seems justified. It is possible that this attachment involves receptor sites on the cell surface that are different than those responsible for

the specific attachment, and that are made available because of the excess of H^+ ions in the medium.

7. Physiological conditions of host cells and IKe adsorption

a. Effect of media: Optimal adsorption of IKe was shown to depend on 0.1M NaCl (see Figure 9). However, the rate of phage attachment measured in 0.1M NaCl solutions is much lower than that in L broth containing NaCl (See Figure 5 and Figure 12). A synthetic medium is preferable to a rich broth medium in the study of adsorption, since in the former, post-adsorption events could be expected to occur at a relatively slower rate thus permitting a more precise measurement of adsorption *per se*. An attempt was therefore made to devise a synthetic medium in which the rate of IKe adsorption is comparable to that in L broth. For this purpose 0.1M NaCl solutions were supplemented as indicated below and the rate of adsorption in each medium determined. Results are shown in Table 6.

The addition of 0.1% dextrose as well as that of L threonine and L leucine increased the rate of adsorption considerably. In fact the rate of adsorption in 0.1M NaCl supplemented with both dextrose and essential amino acids was similar to the rate of adsorption in L broth.

Assays for p.f.u. in adsorption mixtures indicated that neither phage inactivation nor its propagation had occurred in any of the test media during the incubation periods used.

These findings indicate that in addition to the optimal salt concentration, an energy source and essential amino acids are required

Figure 12. Adsorption of IKe in 0.1M NaCl.

Legend: Unadsorbed phage and infective centers were assayed in separate experiments. Cells grown in L broth were filtered, washed and resuspended in chilled 0.1M NaCl. Concentration of cells in adsorption mixtures was 2.6×10^8 v.c./ml (supernatant assay) and 1.2×10^8 v.c./ml (pellet assay). Phage was diluted 100 fold into chilled 0.1M NaCl just before addition to cells. The final concentration of IKe was 5×10^4 p.f.u./ml in supernatant assay and 6×10^5 p.f.u./ml in pellet assay.

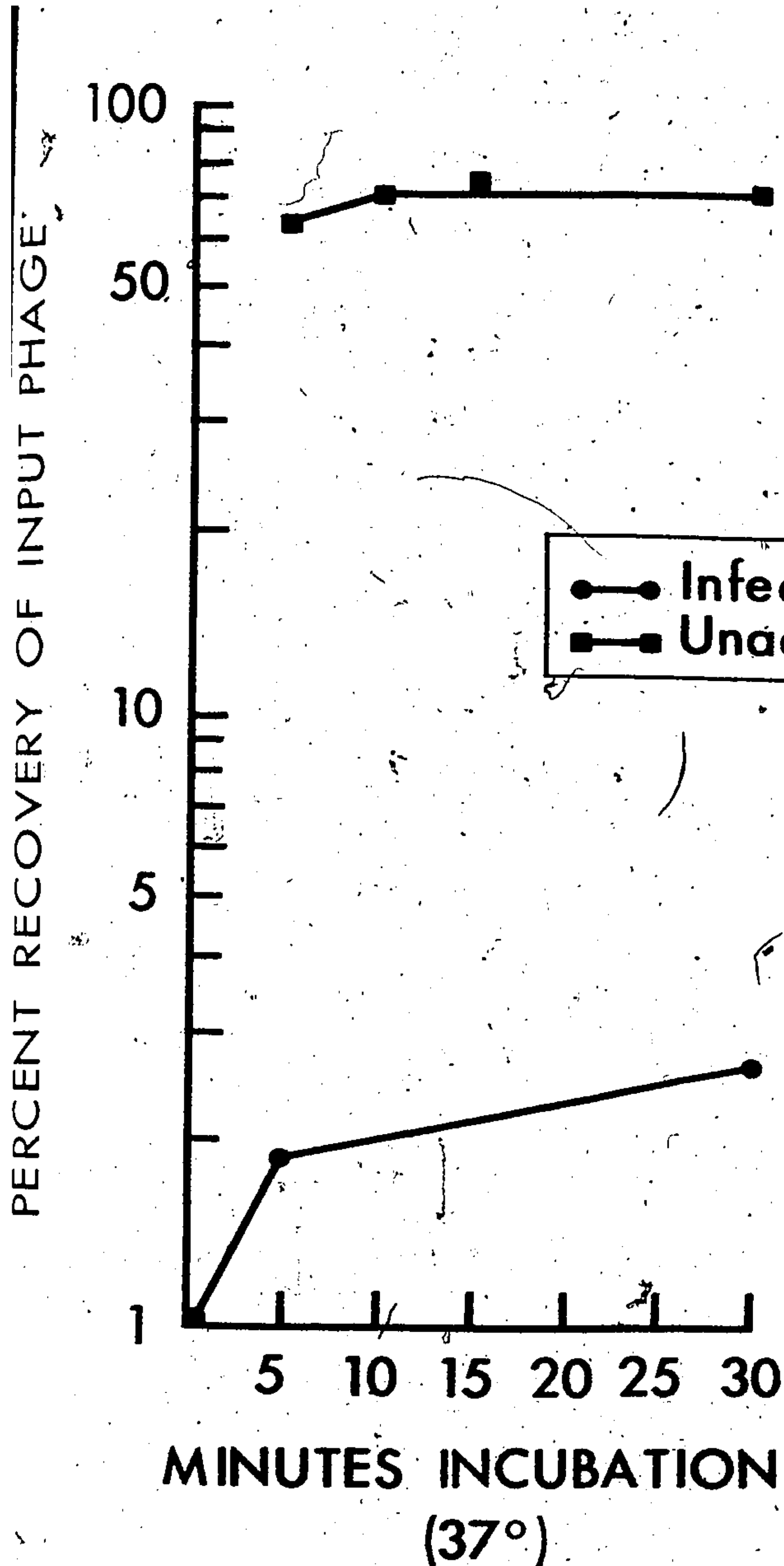

PERCENT RECOVERY OF INPUT PHAGE
100
50
10
5
1
Infective centers
Unadsorbed phage
5
10
15
20
25
30
MINUTES INCUBATION
(37°)

Table 6. Adsorption of IKe in synthetic media.

Media	k (cm^3 min^-)	Infective centers at 30' (% of input)
o.1M NaCl*	2.8×10^{-11}	4.1
NC	3.9×10^{-11}	3.3
NCG	8×10^{-11}	12.4
NCGLT	21×10^{-11}	22
L broth (see p. 21)	23×10^{-11}	

* Adjusted to pH 6.7

Abbreviations

NC = 0.1M NaCl pH 6.7 + 0.002M $CaCl_2$

NCG = 0.1M NaCl pH 6.7 + 0.002M $CaCl_2$ + 0.1% dextrose

NCGLT = 0.1M NaCl pH 6.7 + 0.002M $CaCl_2$ + 0.1% dextrose + L leucine and L threonine (48 μg/ml).

Legend: Host cells grown in L broth were filtered, washed and resuspended in the indicated media. Phage was diluted 1:100 in NC just before addition to cells. Adsorption mixtures contained 2.1×10^8 v.c./ml and 5×10^4 p.f.u./ml. Incubation was at 37°C and supernatants were assayed at 15 and 30 minutes.

by the cells for maximal IKe adsorption.

Adsorption of other DNA phages (T1, T2, T3 T4 and φX174 (26, 66) was reported to be optimal in aqueous solutions containing specific optimal concentrations of salt only.

b. <u>The optimal growth phase</u>: The ability of host cells harvested at different points along their growth curve (see arrows in Figure 13) to adsorb IKe was examined. Results are shown in Table 7.

Maximum adsorption was observed with cells from the mid-logarithmic phase. For all subsequent adsorption experiments cells in this phase of growth were used.

c. <u>Effect of chemical agents</u>:

<u>NaCN and NaN_3</u>: The effect of the metabolic poisons NaCN and NaN_3 on the adsorption of IKe was studied. This was done in an effort to clarify the extent to which adsorption is dependent on the metabolic activity of host cells.

NaN_3 at a concentration of 10^{-2}M was reported to block the irreversible but not the reversible attachment of phage T1 (17). 10^{-2}M NaCN as well as 0.1M sodium arsenate were shown to cause a rapid loss of F pili from the surface of F^+ or Hfr cells (58, 59, 62).

Results of the experiments testing the effect of the metabolic poisons on the attachment of IKe are shown in Table 8. They indicate that blocking of the normal metabolism of host cells by NaCN or NaN_3 causes a significant reduction in their ability to adsorb the phage. This reduction

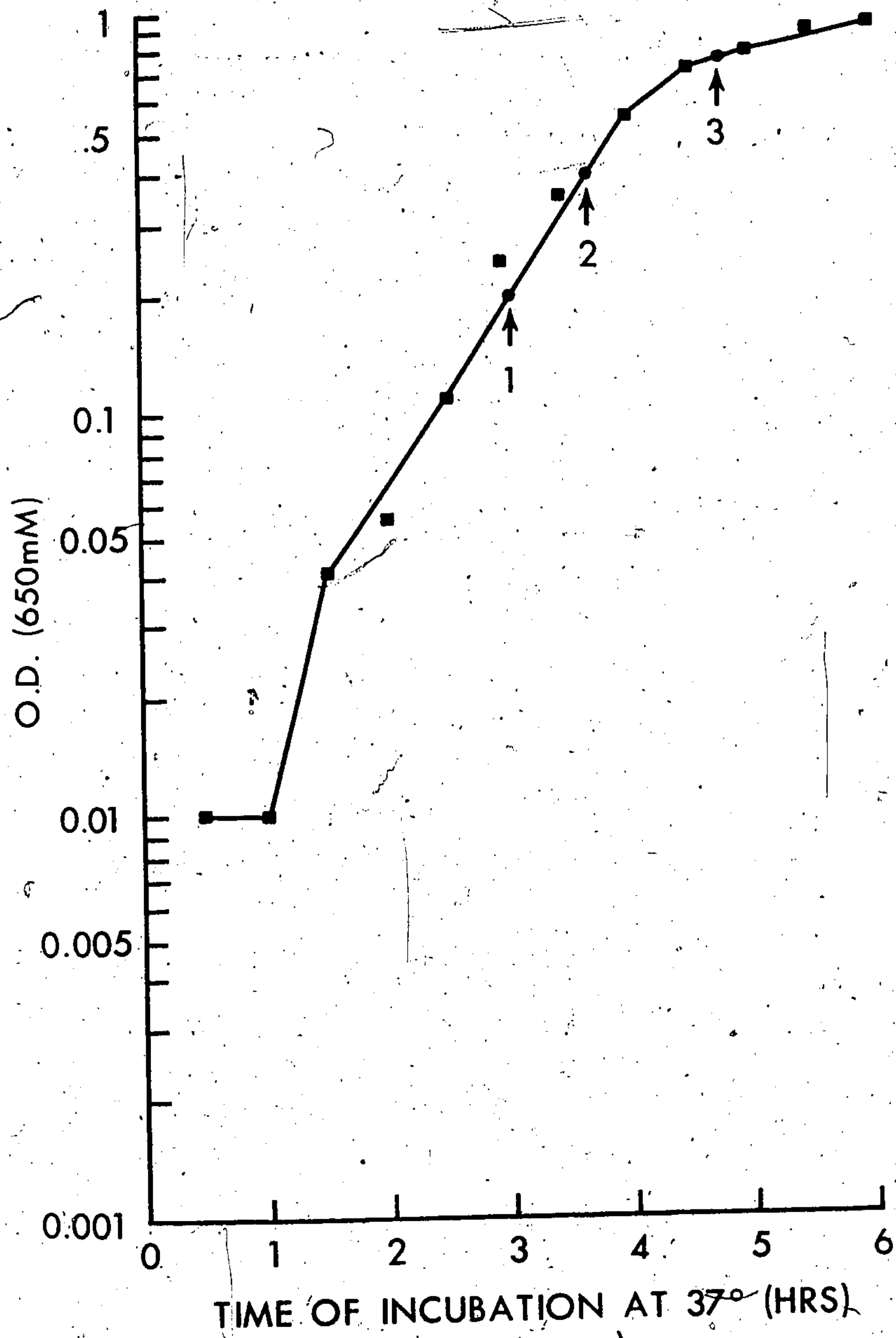
Table 7. The effect of the growth phase of host cells on the adsorption of IKe.

Growth phase (see arrows, fig. 13)	unadsorbed phage (% of input)	Infective centers (% of input)
1	58	13
2	35	20
3	61	10

Legend: JE2571/98-70 cells growing at 37°C were harvested at the optical densities indicated by arrows in fig. 13. Cell concentration in all samples was adjusted to 2.6×10^8 v.c./ml. IKe was added to a final concentration of 7×10^4 p.f.u./ml and incubation was at 37°C for 15'.

Figure 13. A growth curve of JE2571/98-70 in L broth.

Legend: Arrows and closed circles indicate points on the growth curve at which samples were taken (see table 7).



1
.5
0.1
0.05
0.01
0.005
0.001
O.D. (650mM)
0
1
2
3
4
5
6
TIME OF INCUBATION AT 37° (HRS)
1
2
3

is observed with 2 x 10^{-3}M NaCN, a concentration which effectively blocks anaerobic glucose metabolism but does not cause cell death (19) and is enhanced in presence of 10^{-2}M NaCN, a concentration sufficiently high to cause partial cell death.

Chloramphenicol: Experiments described above had demonstrated the dependence of adsorption on the presence of essential amino acids in the adsorption medium (see Table 6). The effect of an inhibitor of protein metabolism on adsorption was therefore of interest.

A concentration of 30 µg/ml of chloramphenicol was reported to effectively inhibit protein synthesis in E. coli cells (51). The effect of preincubation of host cells (for 10 min. at 37°C) with 300 µg/ml chloramphenicol on their ability to adsorb IKe was examined.

Results are shown in Table 8. They suggest that the presence of 300 µg/ml of Cm does not inhibit or reduce adsorption.

These findings are interesting in light of a recent report indicating that post penetration events in F^+ cells infected with M13 are dependent on the presence of essential amino acids in the assay medium. These could not be prevented however by the addition of 180 µg/ml of Cm to the medium (77).

8. The reversibility of IKe adsorption

Under several experimental conditions only a reversible attachment of phages T1, T2 and T7 to their host cells has been shown to occur. Reversibility of attachment was inferred if the phage could be eluted from phage-

Table 8. Effect of growth inhibitors and $ZnSO_4$ on IKe adsorption.

Inhibitor*		Reduction in adsorption (relative to control)	Survival of cells (relative to control)
$ZnSO_4$	10^{-3}M	68%	100%
NaN_3	10^{-2}M	28%	50%
NaCN	$2x10^{-3}$M	35%	100%
NaCN	10^{-2}M	76%	30%
Cm	300 μg/ml	none	50%

Legend: To aliquots of a host culture containing 3×10^8 v.c./ml inhibitors were added at the indicated concentrations. After 5' at room temperature v.c./ml were assayed and phage at a final concentration of 7×10^4 p.f.u./ml added. Incubation was at 30°C for 20'. Unadsorbed phages were assayed by the supernatant method.

* Inhibitors at indicated concentrations did not reduce the plaque forming ability of the phage.

cell complexes after suspension of the complexes in an appropriate elution medium. The majority of the reversibly attached particles (70-90%) could usually be recovered in this medium (16, 17, 27, 28, 66).

The reversibility of the attachment of IKe to its host was tested under conditions reported to permit only reversible binding of the T phages. Results are shown in Table 9. They demonstrate the absence of a reversible adsorption under those conditions. Chilled distilled H_2O was chosen as the eluant since neither adsorption nor inactivation of IKe were found to occur in it under the conditions of the experiment.

C. THE RECEPTOR SITE

1. Effect of Zn^{++} on adsorption

Zn^{++} cations have been reported to inhibit adsorption of several filamentous bacteriophages (64, 79). They have also been reported to inhibit the irreversible step in the adsorption of phage T1 (28) It was suggested that Zn^{++} blocks adsorption of the filamentous phages by interacting with their receptors located on the tips of the F pili.

The effect of Zn^{++} on adsorption of IKe was studied in an attempt to examine the similarities between the receptor sites for IKe and those for pilus-specific filamentous phages.

The results are presented in Table 8. They indicate that $ZnSO_4$ at a concentration of 10^{-3}M did not completely inhibit adsorption of IKe but reduced it by approximately 70%. Adsorption of the filamentous bacteriophage f1 was reported to decrease by 80% in the presence of Zn^{++}

Table 9. The reversibility of IKe adsorption.

Adsorption conditions					Phage lost from supernatant[a,b] (% of input)	Eluted phage (% of input)
v.c./ml	p.f.u./ml	medium	temp.	duration		
3.6×10^8	6×10^4	L broth	$0^{\circ}C$	60'	16	2.2
2.6×10^8	5×10^4	0.1M NaCl	$0^{\circ}C$	95'	22	0.98
1.8×10^8	7×10^4	L broth	$10^{\circ}C$	20'	23	0.14
1.8×10^8	7×10^4	L broth	$45^{\circ}C$	20'	24	0.22
3×10^8	7×10^4	10^{-2}M NaCN in L broth	$37^{\circ}C$	20'	10	0.75
3×10^8	7×10^4	10^{-2}M NaN_3 in L broth	$37^{\circ}C$	20'	23	0.57
3×10^8	7×10^4	10^{-3}M $ZnSO_4$ in L broth	$37^{\circ}C$	20'	20	0.54
1.3×10^8	6×10^4	0.05M NaCl	$37^{\circ}C$	30'	16	< 1%
1.3×10^8	6×10^4	0.5M NaCl	$37^{\circ}C$	30'	1.7	< 1%

Legend: Adsorption mixtures were allowed to incubate as indicated, chilled and centrifuged. Supernatants were assayed for free phage. Pellets were resuspended and held in chilled H_2O for 15'-20' (Phage is stable under these conditions). Suspensions were centrifuged and supernatants assayed for eluted phage.

a. See discussion p.40-41.

b. A direct measurement of adsorbed phage (pellet assay) was avoided since in some of the experiments (e.g. NaCN, NaN_3) cell viability was affected and a true number of i.c. could not be obtained.

at the same concentration (64).

2. Blending of host cells and their ability to adsorb IKe

Mechanical agitation of piliated cells has been reported to result, under certain conditions, in the specific removal of their pili and in a coordinate reduction in their ability to (a) adsorb pilus-specific phages and (b) function as donors (57, 61).

Electron microscope observation on JE2571/98-70 failed to reveal pilus-like structures on the cell surface (14). These findings implied that a structure other than a pilus might be involved in the attachment of phage IKe. The effect of agitation of JE2571/98-70 cells on their ability to bind IKe was therefore of interest.

Results of several experiments are shown in Tables 10 and 11. Table 10 describes the results of pellet assays in which radioactively labelled phage was used at relatively high m.o.i. Table 12 describes the results of supernatant assays in which non-labelled phage was used. This consequently permitted the use of low m.o.i.

Cells were treated with formalin prior to addition of the phage in order to prevent penetration of adsorbed phages and reappearance of any appendages removed by blending (34, 57).

The results shown in Tables 10 and 11 indicate that blending effectively reduced the ability of the F^+ cells to bind M13. Blending however was ineffective in decreasing attachment of IKe and even caused an increase of 2-3 fold in the attachment.

Table 10. The effect of blending on the ability of formalin killed F42+ or RM98+ cells to adsorb M13 or IKe - high m.o.i. experiments.

Exp.#	strain	phage	m.o.i.	Treatment of cells	phage recovered in pellets (% of input)
1.	JE2571/F42	M13	25	unblended	2.9
				blended	1.9
2.	JE2571/F42	M13	25	unblended	1.7
				blended	1.3
3.	JE2571/98-70	IKe	17	unblended	1.1
				blended	4.0
4.	JE2571/98-70	IKe	10	unblended	1.3
				blended	4.0

Legend: JE2571/98-70 and JE2571/F42 were chilled and treated with 3.7% (final concentration) formalin. No viable cells could be recovered 5 mins after addition of formalin. Cultures were blended as described in Materials and Methods (p. 17). Blended and unblended cells were infected with the indicated ^{3}H-thymidine labelled phage Incubation of adsorption mixtures was for 15 min. at 37°C. Phage-cell mixtures were sampled to get total cpm/ml adsorption mixture. Complexes were prepared by the pellet method and the radioactivity associated with pellets (adsorbed phage) counted over Aquasol using a Beckman Liquid Scintillation system.

Table 11. The effect of blending on the ability of formalin killed F42$^+$ and RM98$^+$ cells to adsorb M13 or IKe -- low m.o.i. experiments.

Exp. #	strain	phage	m.o.i.	Treatment of cells	Free phage recovered in supernatant (% of input)
1.	JE2571/F42	M13	0.0007	unblended	77
				blended	99
2.	JE2571/98-70	IKe peak A	0.00015	unblended	83
				blended	68
3.	JE2571/98-70	IKe peak B	0.00012	unblended	95
				blended	62

Legend: Formalin killed cultures (see legend to table 10) were washed free of formalin on sterile Millipore filters and resuspended in L broth. Blended and unblended aliquots of the cultures (p.17) were infected with phage as indicated. Incubation of adsorption mixtures was for 15' at 37°C. Unadsorbed phage was assayed by the supernatant method.

These findings support the electron microscope observation and suggest that a pilus-like receptor is not involved in the attachment of IKe. The significance of the increase in adsorption of IKe after blending is not yet understood. It is of interest however to note that blending was also reported to cause an increase in the attachment of phage T6 to its host cells (61). Bacteriophage T6 has been reported to adsorb to the proteinic fraction of the cell wall (45).

D. PENETRATION

In the infection of host cells, the attachment of IKe is followed by a step which renders the infecting particles insensitive to the proteinase Nagarase. This step takes place at a temperature optimal for the infection (37°C) but not at 0°C. This was demonstrated when phage-cell complexes formed at either 4°C or 37°C were assayed for infective centers before and after exposure to 10mg/ml Nagarase at 37°C. Complexes formed at 37°C but not at 4°C yielded infective centers after treatment with the enzyme.

Tzagoloff and Pratt (79) have interpreted the insensitivity of attached M13 particles to Nagarase as evidence for the penetration of the "Infective principle - the DNA" of the phage into the host cells.

Although the study of adsorption was the main purpose of the current work it was of interest to examine the relationship between adsorption and penetration.

Complexes of IKe and host cells were allowed to form at 37°C. These were separated from unadsorbed phage (see pellet assay) and assayed

for the number of infective centers (comprising attached and penetrated phage). The complexes were then incubated with Nagarase to inactivate unpenetrated phage, centrifuged again and assayed for infective centers (penetrated phage). Complexes formed at 0°C served as controls and did not yield any i.c. after treatment with Nagarase.

Results are shown in Figure 14.

They suggest the following:

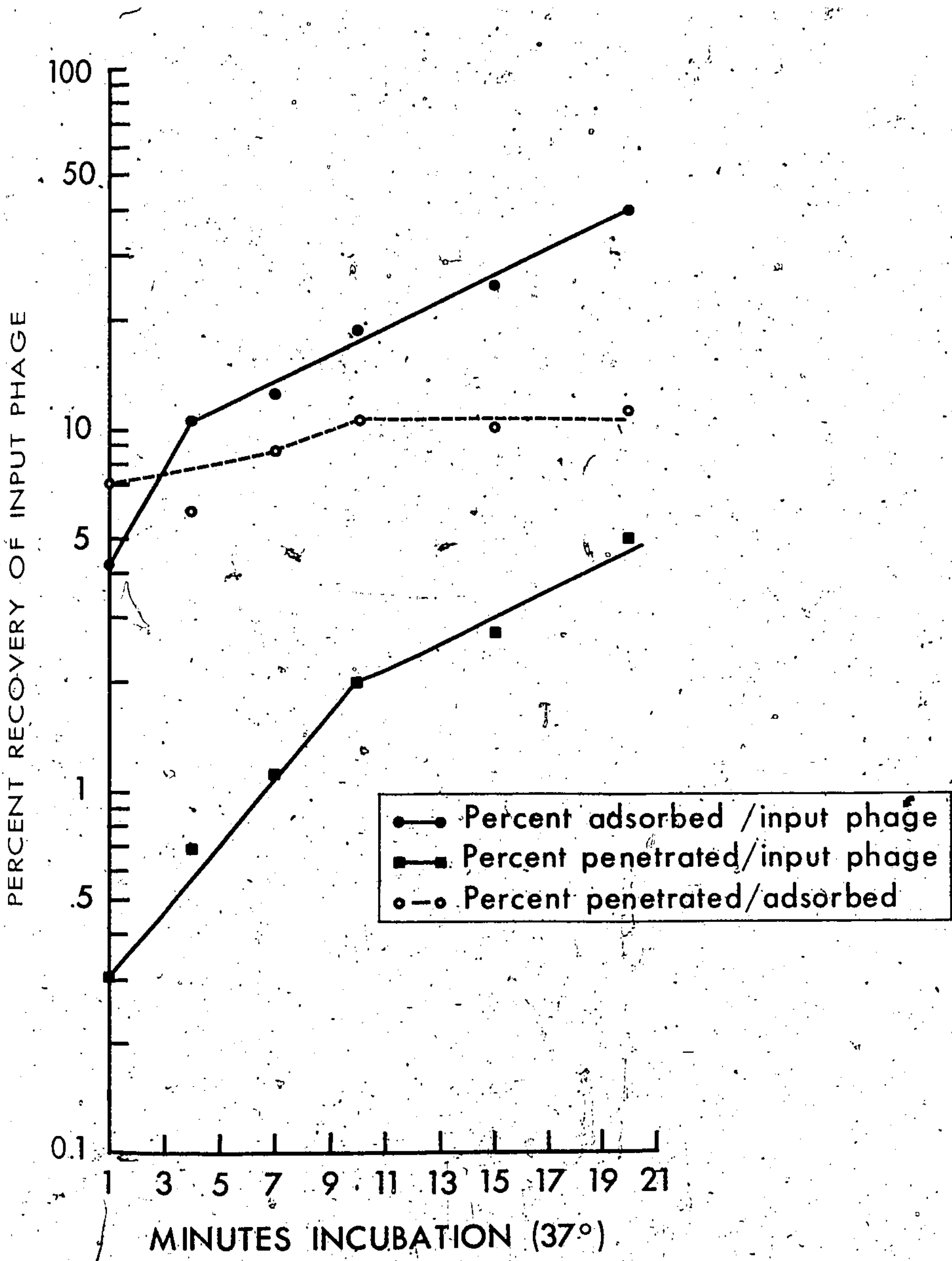
(1) There is a rapid increase in the number of adsorbed particles in the first 4 minutes of incubation at 37°C. The rate of adsorption decreases however after that time and remains constant up to 20' incubation (E.M. observations confirmed these findings (14)).

(2) The rate of penetration is high during the first 10 minutes of incubation. It then decreases and remains constant up to 20' incubation.

(3) The ratio of penetrated: adsorbed phage is about 1:7 in the first 7 minutes of incubation. This ratio then decreases to 1:10 and remains constant thereafter (20' incubation).

Figure 14. Adsorption and penetration of IKe.

Legend: Purified phage-cell complexes (pellet method) were assayed for i.c./ml and v.c./ml. 10 mg/ml of Nagarase were added and incubation at 37°C for 15' followed. Nagarase was removed by centrifugation and the complexes assayed again for v.c./ml and i.c./ml (due to penetrated phage). Adsorption mixtures contained 4×10^9 v.c./ml and 7×10^6 p.f.u./ml. The assays for v.c./ml indicated no change in the number of cells due to assay procedures.



100
50
10
5
1
.5
0.1
PERCENT RECOVERY OF INPUT PHAGE
1 3 5 7 9 11 13 15 17 19 21
MINUTES INCUBATION (37°)
Percent adsorbed /input phage
Percent penetrated/input phage
Percent penetrated/adsorbed

CHAPTER 4

SUMMARY AND CONCLUSIONS

The attachment of phage IKe - Optimal Conditions and the Nature of the binding.

It is generally believed that attachment of bacteriophages to their host cells is the result of the establishment of electrostatic bonds between ionized groups located on the surface of both particles (78, 79). It was demonstrated that every phage-host cell system requires a specific concentration of univalent and/or divalent cations in the media for optimal adsorption to occur (see Table 12). The role of the cations is believed to be in neutralizing repulsing negative electrostatic charges on cell and phage surfaces thus enabling available attracting charges to cause binding between the particles. The specific ionic requirements exhibited by every phage-host cell system are therefore determined by the specific electrostatic charges on the respective particles (65, 66, 79).

Several lines of evidence suggest that adsorption of IKe is also mediated by the formation of electrostatic bonds between the phage and its host cell:

1. Optimal IKe attachment occurs only in media containing 0.1M NaCl.

2. IKe attachment is dependent on pH (tables 3 and 4). It is optimal and does not vary considerably at pH values ranging from 5-8. In the presence of excess H^+ cations (pH 4.2 or lower) however, specific attachment is reduced considerably and a non-specific, non-infectious type of attachment occurs instead. This may be due to the blocking of the specific receptor

sites by the H^+ cations and at the same time the establishment of another set of non-specific attachment sites on the surfaces of the phage and host cell.

3. IKe particles were found to adsorb to non-biological surfaces (membrane filter discs) of net negative charge under the same conditions of ionic concentration required for phage attachment to host cells. These data can actually be conclusive only if additional evidence is provided to ascertain that the same ionic groups are indeed involved in the attachment of IKe to both host cell and the non-biological entity. However, attachment to inorganic surfaces found to occur with the T phages was intensively studied and results indicated that attachment of those phages to the negatively charged inorganic surfaces very likely involved the same ionized groups which bind the phage of its host cell (66, 67).

Thus, attachment of IKe appears similar to that of other bacteriophages in its electrostatic nature. Additional findings seem to suggest however that in the binding of IKe (in contrast to that of other DNA bacteriophages) other factors are involved as well.

1. While high levels of adsorption of T1, ΦX174 or M13 could be obtained in starvation media so long as the ionic environment specifically required by the phage and its host was provided (26, 66, 78, 79) optimal levels of IKe attachment could be obtained only if the mineral salts medium was supplemented with a carbon source and amino acids (see Table 6).

2. Attachment of IKe was found to be more temperature sensitive than

attachment of M13 or the reversible binding of T1. While binding of the latter was obtained at 0°-2°C and increased only approximately 2-fold when temperature was raised to 30°C, attachment of IKe was negligible at 0°C and increased 10-fold when incubation temperature was raised to 30°C. A similar temperature effect was also reported for phage ϕX174 (26, 27, 28, 79).

3. Optimal attachment of phage IKe could be obtained only with host cells in the mid-exponential growth phase.

4. Adsorption of IKe was markedly reduced in the presence of energy poisons (NaCN and NaN_3) in the media. Attachment of M13 and the reversible binding of T1 could take place in the presence of those inhibitors (17, 59).

These findings seem to point out that attachment of IKe is more dependent on the energy metabolism of host cell than the binding of other DNA bacteriophages. Since the penetration of respective host cells by most bacteriophages (or their infective principle - the DNA) has been shown to be an energy-requiring step (20, 48, 55) it is possible that the steps of attachment and penetration in the infection of host cells with IKe are more closely coupled than those in the infection of respective host cells by other bacteriophages. This close relationship was also demonstrated for the irreversible attachment and the penetration of phage T1 (30).

Since a metabolically active cell is a prerequisite for optimal adsorption of IKe, it seems possible that a study of the attachment of IKe to isolated receptor fragments will encounter difficulties which did not

arise in similar studies with other bacteriophages (3, 15, 26, 54, 80, 82).

The reversibility of the attachment

It has been shown that the attachment of the T phages to their host cells occurs in two steps: (1) a reversible binding which under appropriate conditions is rapidly followed by the establishment of (2) an irreversible bond (16, 78). These two consecutive events can be easily separated and distinguished if attachment takes place under conditions which are suitable for the reversible but not the irreversible type of attachment. Reversible attachment has also been demonstrated with ϕX174. Unlike the T phages however, this attachment did not occur as a step preceeding an irreversible binding but rather as an alternative event taking place under conditions which do not permit the irreversible attachment (26).

A reversible binding of the type that exists between the T phages and their host cells could not be demonstrated for IKe under several conditions examined. The possibility however that a weak state of binding between IKe and its host does exist, and that it cannot be detected by the elution methods used in the case of complexes of T phages and their hosts, cannot be rejected without additional experiments. In fact, the discrepancies observed under certain conditions between results obtained by the supernatant assay and those derived from the pellet assay (see p.21-22) rather suggest that IKe particles do not always bind in a manner leading to plaque formation. Alternate methods may be required for detection of

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such particles.

C. The Receptor Site

The results of the blending experiments reported above (see tables 10, 11) as well as electron microscope observations reported elsewhere (14) suggest that unlike the filamentous phages specific for F- or I-like plasmid-bearing cells, IKe, the N plasmid specific phage, does not attach to a microscopically visible pilus. Several lines of evidence obtained in the course of this study seem to indicate however that in some respects, the attachment of IKe to the RM98 bearing cell and the attachment of other filamentous bacteriophages to their respective hosts are similar.

1. Zn^{++}cations have been shown to reduce attachment of the Ff phages to their host cells (64, 79). A similar effect was also demonstrated in the case of IKe and its host cell (see table 8).

It was postulated that Zn^{++}cations prevent attachment of the filamentous bacteriophages by interacting with the receptor molecule and blocking attachment sites. It seems likely therefore that binding of the pilus-specific filamentous bacteriophage and that of IKe to their respective hosts involve similar ionic groups.

2. Tzagoloff et al., reported that exposure of F^+ cells to 45°C greatly reduces their ability to bind the plasmid-specific bacteriophage M13 (79). A similar temperature effect was also demonstrated for the RM98 bearing host and IKe (see table 2). This suggests that a heat-sensitive molecule (probably a protein) is essential for the attachment of both M13 and IKe.

3. Attachment of both M13 and IKe was shown to be optimal at a NaCl concentration of 0.1M, while unaffected by the absence of divalent cations from the medium (Figures 7, 8 and ref. 79).

4. A reversible binding could not be demonstrated for either M13 or IKe (see Table 9 and ref. 79).

These findings suggest that although it is not part of a microscopically visible pilus, the receptor site for IKe may structurally and/or chemically be similar to the pilus-associated receptor. Consequently, the electrostatic bonds involved in the attachment of pilus-specific bacteriophages and of IKe may also be similar.

No direct evidence was obtained in this study regarding the number of IKe receptor sites available on the surface of a RM98 bearing cell. It is generally believed however, that the relative capacity of a specific host cell for phage adsorption is reflected in the rate of attachment which can be expressed by means of a calculated rate constant (45). Judging from the rate constant calculated for the attachment of IKe to *E. coli* JE2571/98-70, it seems that the number of IKe particles that can attach per host cell under optimal conditions is approximately 20 fold lower than the corresponding number reported for the T phages (66). The optimal capacity of the RM98 bearing cell to adsorb IKe appears greater however than that demonstrated for F^+ cells and M13 (79). (This might be partially due to the relative ease with which F pili detach from the cell surface) (45).

These findings as well as other observations reported in this study will be fully understood only if and when additional information concerning the receptor for phage IKe is available. It seems clear, however, from the data presented that IKe attachment does not follow the pattern of one particular class of DNA bacteriophages but rather overlaps with the characteristic properties associated with the attachment of each of these classes (see Table 12).

Recently the isolation of *Pseudomonas aeruginosa* bacteriophages (PO2 and PO4) with the structural features characteristic of the T odd bacteriophages (hexagonal head with a flexible long non-contractile tail) was reported. These phages were shown to infect via a pilus protruding from their host cell surface (8).

It therefore seems justifiable to conclude that structural similarities between bacteriophages do not necessarily imply similar patterns of infection. The mode of attachment which is apparently dependent on the morphology of the phage is obviously determined by other host cell associated factors as well.

Table 12. The attachment of the DNA bacteriophages.

Bacteriophage*	T1	φX174	M13	IKe
Structure	Double-stranded DNA, hexagonal head, and long flexible non-contractile tail	Single-stranded DNA in a tailless incosahedron, with apical capsomeres at the 12 vertices	Single-stranded DNA, filament-like protein envelope	Single-stranded DNA, filament-like protein envelope
Organ and site of attachment	Tip of the tail apparently to the lipoproteinic fraction of the cell wall	One apical capsomere to lypopolysaccharide in cell wall	Tip of phage to tip of a host-cell sex pilus	Tip of phage apparently directly to cell surface
Attachment rate constant	$310X10^{-11}$ ml/min	$10X10^{-11}$ ml/sec	$3 X 10^{-11}$ ml/min	$23 X 10^{-11}$ ml/min
Effect of host cell concentration	Data available from experiments with T4: adsorption linearly dependent (on cell concentration) only up to a specific saturation point	Linear dependence of attachment only up to a saturation point	No saturation point over a wide range of cell concentrations	Linear dependence only up to a certain saturation point
Ionic requirements	Univalent or divalent ions at concentrations of 0.01 or 0.0001M respectively	Divalent cations (0.1 or 0.01M) which cannot be replaced by univalent cations	Optimal attachment at 0.1M of either univalent or divalent cations	0.1M of a univalent salt (studies with NaCl) no requirement for divalent cations
Optimal temperature.	37°C	36°C	30° - 37°C	30°C - 40°C

Attachment at 0°C	No irreversible attachment. Reversible attachment at ½ the optimal rate	No significant attachment.	Attachment at half the optimal rate	Attachment at 1/10 of the optimal rate.
Effect of pH	At pH between 5 and 10 attachment unaffected. Below pH5 and above 10 - it falls rapidly	Maximal attachment (as measured with cell walls) at pH7.5. No attachment at pH below 6.0	Attachment unaffected by pH in the range of 5-8	No considerable difference between attachment at pH5 or 7. A considerable reduction at pH4.2
Adsorption in absence of nutrients	Identical attachment curves in nutrient broth or in 10^{-3}M $MgCl_2$ solutions	High rates of adsorption but no penetration in a starvation buffer	High rates obtained in starvation media.	Attachment rate constant in 0.1M NaCl solutions approximately 10-fold lower than that in L broth
Effect of energy poisons	10^{-2} NaN_3 inhibits irreversible but not reversible attachment	not reported	NaCN causes retraction of pili. Attachment of filamentous phages not inhibited	NaCN and to a lesser extent NaN_3 reduce adsorption at a concentration of 10^{-3}M
Reversibility of attachment	Reversible attachment - intermediate step preceeding the irreversible one	Reversible attachment not as a necessary intermediate step in infection. Obtained only with cell walls and at low ionic concentrations	No reversible attachment detected.	No reversible attachment detected

	T1	ϕX174	M13	IKe
Effect of Zn^{++} cations	In the presence of 10^{-3}M only reversible attachment occurs	Not reported	Attachment reduced by 80% in the presence of 10^{-3}M (f1 the test phage)	Attachment reduced by 68% in the presence of 10^{-3}M
Effect of pre-heating of host cells	at 50°C, irreversible but not reversible attachment is inhibited	Not reported	Exposure to 45°C destroys cell's ability to adsorb the phage	Exposure to 45°C greatly reduces attachment
Effect of blending			Blending removes F pili and reduces attachment	Blending increases attachment 2-3 fold
References	5, 16, 17, 28, 45, 66, 75, 78, 84	5, 26, 45, 65	5, 45, 48, 59, 61, 64, 79	14, 38

*

1. Phage T1 was selected for comparison as representative of the T series, since it was used in most of the early studies concerning the mechanism of adsorption of the T phages. This phage however differs in structure and site of attachment from the T even phages.

2. ϕX174 was selected for comparison because its DNA is single-stranded.

3. M13 was selected for comparison since it is the most extensively studied plasmid-specific filamentous bacteriophage.

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APPENDIX

(List of abbreviations and glossary)

Ap - Ampicillin

Cm - Chloramphenicol

fla - Flagella

hrs - hours

i.c. - Infective centers

min(s) - minutes

m.o.i. - Multiplicity of infection

NCGLT - 0.1M NaCl pH 6.7 + 0.002M $CaCl_2$ + 0.1% dextrose + L leucine and L threonine (48μg/ml)

O.D. - Optical density

p.f.u. - Plaque forming units

pil - Pilus

RTF - Resistance transfer factor

Tc - Tetracycline

v.c. - Viable cells.

The symbols used for designation of the genetic markers of bacterial strains are in accordance with the nomenclature recommendations by Demerec et al (21).

The terms attachment, adsorption and binding were used interchangably, throughout, for the step in bacteriophage infection which unites bacterial cell and bacteriophage and renders the latter sendimentable at a low speed centrifugation.

In this report the criteria for reversibility (or irreversibility) of attachment are the ones used and described in the studies with the T phages only (17, 28, 66).